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STUDIES ON Rh ANTIBODIES

I. Analysis of a Zone Phenomenon in an Rh Antiserum by Splitting the Serum Into Two Fractions by Means of Dialysis

Ernest Witebsky, M.D., and James F. Mohn, M.D.
Buffalo, N. Y.

DIALYSIS for eighteen hours against large volumes of freshly distilled water, resulting in the division of serum into two fractions, the precipitate and the supernatant, has been applied to the study of Rh antisera as reported in a previous communication.[1] The precipitate consisting mostly of globulins which are soluble in physiologic saline solution contains the major portion of the complete (saline) Rh agglutinins. The "supernatant fraction" is a mixture composed of the albumin and a certain amount of the globulins. In this fraction are found anti-Rh antibodies of the incomplete (albumin) variety. The method is of certain practical interest in that rather potent anti-Rh testing reagents can be prepared from sera containing complete (saline) Rh agglutinins of such weak titer that they could not be used for diagnostic purposes. This is accomplished by dissolving the precipitate in a relatively small volume of saline solution. However, the agglutinating power of the "globulin fraction" can be explained not only by concentration of the complete (saline) Rh agglutinins proper but also partly by their separation from the Rh antibodies of the incomplete (albumin) variety which tend to "block" or suppress the saline agglutinins as long as the former also are present. Herein lies the theoretic interest in this procedure, for it seems to prove that the two varieties of Rh antibodies occur in different serum fractions.

Since the original report was made a large number of additional anti-Rh sera have been examined by this method or a slightly modified adaptation of it. Many sera have shown principally the same results when subjected to dialysis. Considering that the method is as crude as it is simple, it was to be expected that not all Rh antisera would follow the same pattern. One serum which was of special interest because it seemed to differ from the regular behavior is the subject of the investigations to be reported in this and the two following communications. It was obtained from an Rh-negative patient who received multiple transfusions of whole blood, the majority of which most probably were Rh positive, stimulating the production of Rh antibodies. A brief case history of this patient follows below:

Mr. Ree had seven hospital admissions between February, 1937, and his death in February, 1946. He suffered primarily from chronic ulcerative colitis which was treated initially by ileostomy and finally by colectomy. During his first six stays in the hospital he received a total of 4,000 c.c. of citrated, whole blood compatible as to the blood group but unknown

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The findings reported in this and the following communications were presented during a meeting in Washington, D. C., on Oct. 20 and 21, 1947, convened at the request of the Surgeon General of the United States Public Health Service for a discussion on the nomenclature of Rh typing serums.

Received for publication, Sept. 7, 1948.

as to the Rh factor. At the time of his final entrance into the hospital to undergo a total colectomy, he received in all 3,000 c.c. of citrated, whole blood belonging to his blood group but irrespective of the Rh type of the various bloods. The last 500 c.c. produced a severe hemolytic transfusion reaction. The analysis of the transfusion reaction carried out in our laboratory revealed the fact that the patient was an Rh-negative individual whose serum contained an Rh antibody. A large quantity of blood was collected from this patient immediately after his decease. Examination of the serum obtained from this specimen showed an Rh antibody of the complete (saline) variety of only weak titer but one of the incomplete (albumin) variety of high titer. However, in no dilution of the patient's serum was agglutination of Rh-positive cells greater than 2-plus macroscopically in the initial investigations carried out. The relatively weak degree of agglutination excluded the use of this serum for practical purposes as a diagnostic reagent. Accordingly, it was decided to dialyze a sample of it.

Two fractions were obtained by dialyzing 100 c.c. amounts of this serum for eighteen hours against two changes of 12 liters each of freshly distilled water in the refrigerator at approximately 4° C. The precipitate, to be referred to as the "globulin fraction," was separated by centrifugation from the remaining dialysate, to be referred to as the "supernatant fraction." The "globulin fraction" was dissolved in 10 c.c. of 0.9 per cent saline solution corresponding to one-tenth the amount of the original serum specimen (a ten times concentration) and lyophilized in small ampules containing 1.0 c.c. each. When needed, they were dissolved by the addition of 1.0 c.c. of distilled water. The salt-free "supernatant fraction" was also lyophilized and when needed was dissolved in physiologic saline solution. The experiments recorded in this paper all refer to the two serum fractions obtained in this manner.

In order to compare the relative distribution of the complete (saline) and incomplete (albumin) Rh antibodies in the two serum fractions with that in the native (untreated) serum, the first experiment was carried out in two parts. In Part I saline was used as the medium for preparing the dilutions of the serum and its fractions and for suspending the Rh-positive test cells, while in Part II, undiluted, normal, adult, human serum of Group O was used as a diluent. Experiment I itself was carried out in the following manner:

Decreasing amounts of native serum (Ree) and its two fractions, volume 0.05 c.c., were mixed with 0.05 c.c. of a 2 per cent suspension of homozygous Rh_1 (CDe/Ce) cells belonging to blood Group O. The tubes were shaken thoroughly. After standing for one hour at room temperature they were centrifuged at approximately 1,500 revolutions per minute for two minutes and read macroscopically for agglutination as recorded in Table I.

This experiment shows that the complete (saline) Rh antibodies, which were very weak from the start, are somewhat concentrated in the "globulin fraction" corresponding to previous experiences. The "supernatant fraction" does not contain any complete (saline) anti-Rh agglutinins. When saline is replaced as a diluent by undiluted, normal, adult, human serum, incomplete (albumin) Rh agglutinins of considerable titer become apparent in the native serum. In this experiment, however, the degree of agglutination of Rh-positive test cells does not exceed the 2-plus stage in any of the dilutions tested. A zone phenomenon in the middle of the titration can be recognized. In contrast to the native serum, the "globulin fraction" produces a 4-plus agglutination of the Rh-positive cells which is stronger than might be expected even if it is realized that this fraction corresponds to a ten times concentration of the original native serum. This "globulin fraction" also differs from "globulin fractions" previously described inasmuch as it contains Rh antibodies of the incomplete (albumin) variety reacting much stronger if undiluted serum is used as a diluent instead of saline solution.

The second fraction, "the supernatant," reveals a prozone phenomenon which is absent both in the native serum and in the "globulin fraction." One

TABLE I. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY SERUM (REE) AND ITS TWO FRACTIONS ("GLOBULIN AND SUPERNATANT")

DILUENT	PART I 0.9% SALINE			PART II UNDILUTED HUMAN SERUM		
SERUM (REE)	A NATIVE	B GLOBULIN	C SUPER-NATANT	A NATIVE	B GLOBULIN	C SUPER-NATANT
1. Undiluted	+	+ +	–	+ +	+ + + +	–
2. 1:2	±	+	–	+ +	+ + + +	±
3. 1:4	–	+	–	+ +	+ + + +	±
4. 1:8	–	±	–	+	+ + + +	+
5. 1:16	–	±	–	+	+ + + +	+ +
6. 1:32	–	±	–	+	+ + + +	+ +
7. 1:64	–	+	–	+ +	+ + +	+
8. 1:128	–	+	–	+ +	+ +	+
9. 1:256	–	±	–	+ +	+ +	+ +
10. 1:512	–	–	–	+ +	+ +	+ +
11. 1:1,024	–	–	–	+ +	+	+ +
12. 0	–	–	–	–	–	–

–, No agglutination; ±, Faint agglutination; +, Slight agglutination; + +, Marked agglutination; + + +, Strong agglutination; + + + +, Very strong agglutination.

could readily visualize how the combined action of the two separate serum fractions could result in the type of agglutination that is shown by the native serum.

Inasmuch as the experiment shown in Table I did not reach the end point of agglutination and because of the zone phenomenon observed, a second experiment was carried out as follows:

Decreasing amounts of the native serum (Ree) and its two fractions respectively, volume 0.05 c.c., were mixed with 0.05 c.c. of a 2 per cent suspension of homozygous Rh_1 (CDe/Ce) Group O cells. The mixtures were allowed to remain for one hour at room temperature and then were spun down. The resulting agglutinations as read macroscopically are shown in Table II.

TABLE II. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY THE INCOMPLETE ANTI-RH ANTIBODIES OF SERUM (REE) AND OF ITS TWO FRACTIONS—END POINT TITRATIONS

SERUM (REE)	A NATIVE	B GLOBULIN	C SUPERNATANT
1. Undiluted	+ + +	+ + + +	–
2. 1:2	+ +	+ + + +	–
3. 1:4	+ +	+ + + +	±
4. 1:8	+ +	+ + + +	+
5. 1:16	+	+ + + +	+ +
6. 1:32	+	+ + + +	+ +
7. 1:64	+ +	+ + + +	+ + +
8. 1:128	+ +	+ + +	+ + +
9. 1:256	+ + +	+ + +	+ + +
10. 1:512	+ + +	+ + +	+ + +
11. 1:1,024	+ + +	+ + +	+ +
12. 1:2,048	+ + +	+ + +	+ +
13. 1:4,096	+ + +	+ + +	+ +
14. 1:8,192	+ +	+ +	+
15. 1:16,384	+ +	+ +	±
16. 1:32,768	+ +	+ +	–
17. 1:65,536	+	+ +	–
18. 1:131,072	+	+ +	–
19. 1:262,144	+	+	–
20. 1:524,288	±	+	–
21. 1:1,048,576	–	±	–
22. 1:2,097,152	–	–	–
23. 1:4,194,304	–	–	–
24. 0	–	–	–

All dilutions made with undiluted, normal, adult, human serum of Group O.

Experiment II again demonstrates the fact that the native serum upon titration exhibits a zone phenomenon most obviously in dilutions of 1:16 or 1:32, reaching an end point titer of approximately 260,000.* The "globulin fraction" reveals the presence of an incomplete (albumin) variety of Rh antibody of considerable strength decreasing in potency in a straight line with an end point of approximately 1:500,000* without showing any zone phenomenon. No agglutination occurs in the undiluted "supernatant fraction." As this fraction is diluted further an incomplete (albumin) anti-Rh antibody of increasing strength becomes apparent until a dilution of 1:512 is reached, whereafter it decreases, reaching an end point titer of approximately 1:8,000.*

The preceding experiments focused attention on the peculiar behavior of the "supernatant fraction" when titrated in undiluted, normal, adult, human serum. Was the prozone phenomenon due to surplus inhibition or to the activity of an antibody truly blocking in nature? When Wiener[2] first described his Rh "blocking antibody" ("incomplete" Rh antibody of Race[3]), he demonstrated its presence by its "blocking" effect on the Rh saline agglutinin (complete Rh antibody). However, experiments by Diamond and associates[4-6] and Wiener[7-9] revealed that the so-called "blocking" antibody did not "block" at all if as a diluent saline solution were replaced by 20 per cent bovine albumin solution or undiluted human serum. Use of these latter substances as diluents resulted in agglutination of Rh-positive cells treated with such a "blocking" or "incomplete" antibody. In the case under discussion, undiluted, normal, adult, human serum was used as a diluent and still a prozone phenomenon was observed.

The blocking effect of the "supernatant fraction" was tested in Experiment III following a similar order of experiment as used in an earlier communication.[1] The cell suspension and the dilutions of the "supernatant fraction" were all made in undiluted human serum instead of in physiologic saline solution. The experiment itself was carried out thusly:

Decreasing amounts of (A) "the supernatant fraction" of serum (Ree) and (B) the "supernatant fraction" of a normal, adult serum, used in this experiment as a control, volume 0.15 c.c., were mixed with 0.05 c.c. of a 6 per cent suspension of homozygous Rh_1 (CDe/Ce) cells belonging to blood Group O. The experiment was set up in duplicate (Part I and Part II). After incubation for one hour at room temperature, the following anti-Rh sera were added: to each tube in Part I, 0.05 c.c. of undiluted "globulin fraction" of serum (Ree); to each tube in Part II, 0.05 c.c. of undiluted anti-D (Rh_0) serum (Dad) containing an Rh antibody of the incomplete (albumin) variety. The tubes after being shaken well were kept for an additional hour at room temperature, then centrifuged and read macroscopically for agglutination. The results obtained are recorded in Table III.

It can be seen that the "supernatant fraction" of serum (Ree) does indeed inhibit or block its own "globulin fraction," thus preventing the "globulin fraction" from agglutinating the Rh-positive test cells. It also prevents the agglutination of Rh-positive cells by anti-Rh agglutinins of the incomplete (albumin) variety contained in serum (Dad) (Part II) as compared with the "supernatant fraction" of the normal serum used as a control.

In Experiment III, decreasing amounts of the "supernatant fraction" showing the blocking phenomenon were mixed with constant amounts of the "globulin

*Inasmuch as pipettes were not changed with each dilution these figures should not be taken as absolute values.

TABLE III. INHIBITORY EFFECT OF THE "SUPERNATANT FRACTION" OF SERUM (REE) ON THE AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY RH AGGLUTININS OF THE INCOMPLETE VARIETY

SUPERNATANT FRACTIONS	PART I GLOBULIN FRACTION (REE)		PART II ANTI-RH SERUM (DAD)	
	A SERUM (REE)	B NORMAL SERUM	A SERUM (REE)	B NORMAL SERUM
1. Undiluted	±	+ + +	−	+ + +
2. 1:3	+	+ + + +	±	+ + + +
3. 1:9	+ +	+ + + +	+	+ + + +
4. 1:27	+ +	+ + + +	+ +	+ + + +
5. 1:81	+ +	+ + + +	+ +	+ + + +
6. 0	+ + + +	+ + + +	+ + + +	+ + + +

All dilutions made with undiluted, normal, adult, human serum of Group O.

fraction" and with another anti-Rh serum containing incomplete (albumin) Rh antibodies. The next experiment to be reported reveals the blocking effect of this "supernatant fraction" of serum (Ree) when it is added in a constant amount to decreasing amounts of its own "globulin fraction" and its own "supernatant fraction." The experiment was performed as follows:

Decreasing amounts of (A) the "globulin fraction" obtained from anti-Rh serum (Ree) and (B) the "supernatant fraction" obtained from anti-Rh serum (Ree), volume 0.05 c.c., were mixed in Part I with 0.1 c.c. of undiluted "supernatant fraction" of serum (Ree), and in Part II with 0.1 c.c. of undiluted "supernatant fraction" of a normal serum. After the tubes were shaken, 0.05 c.c. of a 5 per cent suspension of Rh_2 (cDE/c) Group O cells was added to each. The tubes after remaining one hour at room temperature were centrifuged at 1,500 revolutions per minute for two minutes; the resultant agglutinations were read macroscopically

TABLE IV. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY THE "GLOBULIN AND SUPERNATANT FRACTIONS" OF SERUM (REE) AFTER BEING MIXED WITH THE "SUPERNATANT FRACTION" OF SERUM (REE)

FRACTIONS OF ANTI-RH SERUM (REE)	PART I SUPERNATANT FRACTION OF SERUM (REE)		PART II SUPERNATANT FRACTION OF NORMAL SERUM	
	A GLOBULIN	B SUPERNATANT	A GLOBULIN	B SUPERNATANT
1. Undiluted	+ + +	−	+ + + +	−
2. 1:2	+ + +	−	+ + + +	−
3. 1:4	+ +	−	+ + + +	±
4. 1:8	+ +	−	+ + + +	+
5. 1:16	+	−	+ + + +	+
6. 1:32	±	−	+ + +	+ +
7. 1:64	−	−	+ + +	+ +
8. 1:128	−	−	+ + +	+ + +
9. 1:256	−	−	+ + +	+ +
10. 1:512	−	−	+ +	+
11. 1:1,024	−	−	+ +	+
12. 1:2,048	−	−	+ +	±
13. 1:4,096	−	−	+ +	−
14. 1:8,192	−	−	+ +	−
15. 1:16,384	−	−	+	−
16. 1:32,768	−	−	+	−
17. 1:65,536	−	−	±	−
18. 1:131,072	−	−	±	−
19. 1:262,144	−	−	−	−
20. 1:524,288	−	−	−	−
21. 1:1,048,576	−	−	−	−
22. 1:2,097,152	−	−	−	−
23. 0	−	−	−	−
24. 0 + 0.1 c.c. serum diluent	−	−	−	−

All dilutions made with undiluted, normal, adult, human serum of Group O.

and recorded in Table IV. In this experiment all dilutions and cell suspensions were prepared with undiluted, normal, adult, human serum of Group O.

When a constant amount of the undiluted "supernatant fraction" of Rh antiserum (Ree) is added to decreasing amounts of its own "globulin fraction," a marked suppression of the agglutination of the Rh-positive test cells results, reducing the titer of its own "globulin fraction" from 32,000 to 16. Furthermore, the undiluted "supernatant fraction" of serum (Ree) when added in constant amount to decreasing amounts of the same "supernatant fraction" of serum (Ree) itself, completely suppresses and prevents the agglutination of the Rh-positive cells by the incomplete (albumin) Rh antibody contained in the greater dilutions of this "supernatant fraction." In contrast, the addition of the "supernatant fraction" of a normal serum fails to prevent the agglutination of the Rh-positive test cells by the "globulin fraction" and the "supernatant fraction" of serum (Ree).

In the experiments described thus far, the blocking effect of the "supernatant fraction" of the anti-Rh serum (Ree) was tested against its own "globulin fraction," which contains a very potent incomplete (albumin) Rh antibody, and against one additional anti-Rh serum (Dad) containing the same type of Rh antibodies but of lower titer. What effect would the "supernatant fraction" of serum (Ree) have on anti-Rh antibodies of the complete (saline) variety? To find the answer to this question, two anti-Rh sera with antibodies of the complete (saline) variety and an additional anti-Rh serum with antibodies of the incomplete (albumin) variety were tested in Experiment V. All of the dilutions and the cell suspension were made with undiluted, normal, adult, human serum of Group O. This experiment was carried out in the following manner:

Decreasing amounts of (A) anti-Rh globulin (Dib), containing Rh antibodies of the complete (saline) variety, (B) anti-Rh serum (And), containing Rh antibodies of the complete (saline) variety, and (C) anti-Rh serum (Dad), containing Rh antibodies of the incomplete (albumin) variety, volume 0.05 c.c., were mixed in Part I with 0.1 c.c. of undiluted "supernatant fraction" of serum (Ree) and in Part II with 0.1 c.c. of undiluted "supernatant fraction" of a normal serum. Each tube received 0.05 c.c. of a 5 per cent suspension of Rh_2 (cDE/c) cells belonging to the blood Group O. Following the addition of the test cells, the mixtures were incubated for one hour at room temperature and were then centrifuged. The resulting agglutinations are recorded in Table V.

TABLE V. BLOCKING EFFECT OF "SUPERNATANT FRACTION" OF SERUM (REE) ON AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY THREE ANTI-RH SERA

	PART I SUPERNATANT FRACTION OF SERUM (REE)			PART II SUPERNATANT FRACTION OF NORMAL SERUM		
ANTI-RH SERA	A GLOBULIN (DIB)	B SERUM (AND)	C SERUM (DAD)	A GLOBULIN (DIB)	B SERUM (AND)	C SERUM (DAD)
1. Undiluted	–	–	–	++++	+++	++
2. 1:2	–	–	–	++++	+++	+
3. 1:4	–	–	–	+++	++	±
4. 1:8	–	–	–	+++	++	–
5. 1:16	–	–	–	++	+	–
6. 1:32	–	–	–	+	±	–
7. 1:64	–	–	–	+	–	–
8. 1:128	–	–	–	±	–	–
9. 1:256	–	–	–	–	–	–
10. 0	–	–	–	–	–	–

A, Globulin (Dib) contains anti-Rh antibodies of the incomplete (albumin) variety.
B, Serum (And) contains anti-Rh antibodies of the complete (saline) variety.
C, Serum (Dad) contains anti-Rh antibodies of the incomplete (albumin) variety.

It can be seen from this experiment that the "supernatant fraction" of serum (Ree) completely suppresses the agglutination of Rh-positive cells by anti-Rh agglutinins irrespective of whether they are of the incomplete or the complete variety. The "supernatant fraction" prepared from a normal human serum does not prevent the agglutination of the Rh-positive cells by any of the anti-Rh sera tested.

DISCUSSION

Zone phenomena frequently have been seen upon quantitative titration of Rh antisera. Taylor, Race, Prior, and Ikin[10] reported an anti-Rh-containing serum which when used undiluted caused, according to their interpretation, the agglutination of cells classed as strongly positive reactors to Rh antibodies but failed to agglutinate weaker cells. Upon serial dilutions of this serum these weakly reacting cells also were agglutinated. The authors suggested at this time that the titration method should be applied to sera for the detection of Rh antibodies.

Attention was called by Levine[11] to the fact that prozone phenomena were occasionally observed in anti-Rh sera. He warned that such sera should not be used for routine Rh typing. The same author, with Waller,[12] in additional experiments carried out on sera exhibiting prozones, reported that this phenomenon could be explained by the fact that such sera contained a mixture of complete (saline) and incomplete (albumin) Rh agglutinins. The latter antibody acted as a "blocking antibody" preventing the agglutination of Rh-positive cells in the first few dilutions. By absorption with Rh-positive cells this prozone could be eliminated, apparently due to the removal of the incomplete (albumin) type of Rh antibody.

After the investigations of various authors had shown that the "blocking" antibody of Wiener, or the "incomplete" antibody of Race, could be demonstrated directly when undiluted human serum or 20 per cent bovine albumin replaced saline solution as the diluent, Levine[13] reported that quantitative studies on the "direct reaction" of this so-called "blocking" antibody revealed the presence of a prozone phenomenon in certain instances. He raised the question whether there were not two varieties of "blocking" antibodies.

Hattersley and Fawcett[14] reported three instances of Rh antisera showing prozones when titrated in undiluted human serum and tested against Rh-positive cells suspended in 30 per cent bovine albumin. The prozones observed by them did not appear at room temperature but only following incubation of the serum-cell mixtures for one hour at 37° C. This prozone occurred only with cells of the subtype Rh_0 but not with Rh′ or Rh″ cells. If the tubes were centrifuged immediately after the titration was completed the prozone phenomenon failed to appear. As a result of this observation Hattersley[15] suggested a modification of the technique of routine Rh typing, using the readily available Rh antisera containing Rh agglutinins of the incomplete (albumin) variety and centrifuging at high speed without preliminary incubation in order to eliminate falsely negative reactions as a result of the prozone phenomenon. It is interesting to note that the prozone phenomenon increased in Hattersley and Fawcett's case 3 following stimulation of the patient with small injections of Rh-positive blood belonging to the subtype Rh_1.

SUMMARY AND CONCLUSIONS

The method of splitting anti-Rh sera into two fractions by dialysis, namely the precipitate (globulins) and the supernatant (mixture of albumin and globulins), has been applied to the serum of an Rh-negative patient (Ree) who produced Rh antibodies following multiple transfusions of Rh-positive blood over a period of several years. The comparison of the untreated serum with its two fractions led to the following observations:

1. The native, untreated serum when tested for Rh antibodies, using undiluted human serum as a diluent, contained Rh agglutinins of the incomplete (albumin) variety up to a titer of approximately 260,000. However, definite zones of agglutination became apparent upon quantitative titration.

2. Marked agglutination of Rh-positive cells decreasing in strength in a straight line upon dilution was produced by the "globulin fraction." No zone phenomenon was observed with this fraction.

3. The second fraction, the "supernatant," showed a strong prozone phenomenon in the first few dilutions. By "combining" the two fractions, the "globulin" and the "supernatant," the irregular zone phenomenon of the native, untreated serum can be understood.

4. The "supernatant fraction" of serum (Ree) blocks the agglutination of Rh-positive cells by both the complete (saline) and the incomplete (albumin) varieties of Rh antibodies. This is in contrast to the blocking effect of the Rh incomplete (albumin) antibody which inhibited the agglutination of Rh-positive cells by the complete (saline) Rh antibody only but induced agglutination of Rh-positive cells by its own action when saline solution was replaced by undiluted normal serum or albumin solution.

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STUDIES ON Rh ANTIBODIES

II. The Demonstration of a Third Type of Rh Antibody With Blocking Properties

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THE preceding communication[1] described experiments carried out on an anti-Rh serum obtained from an Rh-negative patient who had received multiple transfusions of Rh-positive blood. This serum showed a peculiar zone phenomenon upon quantitative titration. When divided into two fractions by dialysis, the precipitate (the "globulin fraction") and the supernatant (the "supernatant fraction"), the "globulin fraction" produced agglutination of Rh-positive cells decreasing in potency in a straight line following the serial dilutions without exhibiting a zone phenomenon. In contrast the "supernatant fraction" showed a definite prozone phenomenon which was not evident in the native serum itself.

Prozone phenomena have been encountered frequently during Rh antibody investigations as mentioned in the preceding paper.[1] The experiments to be reported in this second communication deal with the problem of whether the prozone phenomenon found in the "supernatant fraction" of serum (Ree) is due to a surplus inhibition (surplus of Rh antibodies) or is caused by the presence of a peculiar antibody exhibiting a blocking effect which, in contrast to the "blocking" antibody described by Wiener,[2] manifests itself even when undiluted, normal, adult, human serum is used as a diluent.

Assuming that we are dealing with a true antibody effect and not a surplus inhibition, Rh-positive cells treated with such an antibody should remain blocked although subjected to multiple washings with physiologic saline solution or undiluted, normal, human serum. On the other hand, if the zone phenomenon were due to a surplus of Rh antibodies, multiple washings of the sensitized cells should result in a removal of this surplus of antibodies thus causing agglutination to occur. The experiments to be reported herein deal with this problem. From the technical point of view two different procedures were involved in each experiment: one, in which the Rh-positive cells were treated with the "supernatant fraction" of serum (Ree) followed by a series of thorough washings of these blood cells, and the second, which consisted of the actual testing of the sensitized cells by means of the addition of anti-Rh sera containing different varieties of Rh antibodies.

The first experiment well illustrates the principal procedures employed. It was performed in order to determine whether or not the "supernatant frac-

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tion'' of serum (Ree) contained an antibody which would sensitize Rh-positive cells and thus prevent their agglutination when subsequently mixed with anti-Rh serum. Experiment I was carried out as follows:

I. *Method of Sensitization of Rh-Positive Cells.*—

1. 0.5 c.c. of a 10 per cent suspension of Rh_2 (cDE/c) cells belonging to blood Group O was added to each of ten tubes in two rows of five tubes each.
2. To Row (A), increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, and (5) 1:81 of the ''supernatant fraction'' of serum (Ree) were added in a volume of 1.5 c.c. per tube. A total volume of 2.0 c.c. was obtained in all tubes, the only difference being the amount of the ''supernatant fraction'' present in each tube.
3. The tubes were shaken well and kept for thirty minutes in the refrigerator at 4° C.
4. After centrifuging the tubes for ten minutes at 4,500 revolutions per minute, the supernatant fluids were aspirated and discarded.
5. The sediments, consisting of packed red blood cells, were washed thoroughly three times using 5.0 c.c. of ice-cold 0.9 per cent saline solution per tube.*
6. After the third washing all of the saline solution was removed as completely as possible by aspiration.
7. 0.5 c.c. of undiluted, normal, adult, human serum was added to each tube and the sedimented cells were resuspended completely in the serum.
8. A parallel Row (B) was set up under identical experimental conditions (steps 1-7), the only difference being that the ''supernatant fraction'' of a normal serum was used (control).

II. *Method of Testing the Sensitized Rh-Positive Cells for Agglutination With Anti-Rh Sera.*—0.05 c.c. of the cell suspensions was removed from each of the tubes of Row (A) and Row (B) and pipetted into a new series of small tubes. The experiment was carried out in duplicate. To each tube in Part I, 0.05 c.c. of undiluted anti-D (Rh_o) serum containing Rh agglutinins of the complete (saline) variety was added, and to each tube in Part II, 0.05 c.c. of undiluted anti-D (Rh_o) serum containing Rh agglutinins of the incomplete (albumin) variety was added. After being allowed to stand for thirty minutes at room temperature, the tubes were spun down for two minutes at 1,500 revolutions per minute. The resulting agglutinations are seen in Table I.

TABLE I. AGGLUTINATION BY ANTI-RH SERA OF RH-POSITIVE CELLS TREATED WITH THE ''SUPERNATANT FRACTION'' OF SERUM (REE)

SUPERNATANT FRACTIONS	PART I ANTI-D (Rh_o) AGGLUTININS COMPLETE (SALINE) VARIETY		PART II ANTI-D (Rh_o) AGGLUTININS INCOMPLETE (ALBUMIN) VARIETY	
	A SERUM (REE)	B NORMAL SERUM	A SERUM (REE)	B NORMAL SERUM
1. Undiluted	–	++++	–	++++
2. 1:3	–	++++	+	++++
3. 1:9	–	++++	+	++++
4. 1:27	+	++++	++	++++
5. 1:81	++	++++	+++	++++

All dilutions made with undiluted, normal, adult, human serum of Group O.
–, No agglutination.
±, Faint agglutination.
+, Slight agglutination.
++, Marked agglutination.
+++, Strong agglutination.
++++, Very strong agglutination.

*Physiologic saline solution was used for all of the dilutions and washings as previous experiments revealed that there was no difference in the outcome of the experiments irrespective of whether saline or undiluted, normal, adult, human serum was used.

According to Experiment I, Rh-positive cells treated with the "supernatant fraction" of serum (Ree) fail to be agglutinated upon the addition of Rh antibodies of both the complete (saline) and the incomplete (albumin) variety depending upon the amount of the "supernatant fraction" of the serum (Ree) used for the sensitization of these cells. The complete (saline) type of Rh agglutinin seems to be blocked more easily than the incomplete (albumin) type. In contrast, the "supernatant fraction" obtained from a normal, adult, human serum does not exhibit any blocking properties, indicating the specificity of the effect observed.

The results of this experiment were corroborated in many others carried out in an identical or similar manner and seem to suggest that the prozone phenomenon observed in the "supernatant fraction" of serum (Ree) is due to a peculiar type of Rh antibody rather than to the presence of a surplus of Rh antibodies which should have been removed by the multiple washings.

The question arose whether the inhibition of agglutination shown in the first experiment could be reproduced with an Rh antibody of the known incomplete (albumin) variety previously described by Race[3] and Wiener,[2] or whether the blocking phenomenon was caused by a different third type of Rh antibody present in the "supernatant fraction" of serum (Ree).

In an attempt to answer this problem, an experiment was carried out on a qualitative basis comparing the "supernatant fraction" of serum (Ree) with the "supernatant fraction" of anti-Rh serum (Fis) known to contain Rh agglutinins of the incomplete (albumin) variety. Experiment II was performed as follows:

I. *Method of Sensitization of Rh-Positive Cells.*—

1. 0.5 c.c. of a 10 per cent suspension of heterozygous Rh_1 (CDe/ce) cells belonging to blood Group O was added to each of three tubes.
2. 0.5 c.c. of undiluted "supernatant fractions" was added to each tube as follows:
   Tube (A): "Supernatant fraction" of serum (Ree).
   Tube (B): "Supernatant fraction" of serum (Fis).
   Tube (C): "Supernatant fraction" of a normal, adult, human serum (control).
3. The three tubes were shaken well and kept for thirty minutes in the refrigerator at 4° C. Following this incubation period, examination of the tubes revealed agglutination in tube (B) with the "supernatant fraction" of serum (Fis) known to contain incomplete (albumin) Rh agglutinins. No visible agglutination was detectable in the other two tubes (A) and (C).
4. The tubes were centrifuged for ten minutes at 4,500 revolutions per minute and the supernatants were aspirated and saved for further testing as will be described in a later experiment (Experiment III).
5. The cell sediments were washed thoroughly three times, using 5.0 c.c. of ice-cold physiologic saline solution for each tube. When saline solution was added the first time, the packed cells in tubes (A) and (C) completely dispersed showing no agglutination. However, the cells in tube (B), which had been treated with the incomplete (albumin) Rh agglutinins of the "supernatant fraction" of serum (Fis), again showed definite agglutination. This clumping was completely broken up by vigorous shaking and inverting of the tube. Some agglutination still occurred in tube (B) following the second washing but to a much lesser degree than at the time of the first washing. When the cells were washed a third time, no visible agglutination was noticeable.

6. After the third washing and centrifugation the saline solution was removed as thoroughly as possible by aspiration.
7. 0.5 c.c. of undiluted, normal, adult, human serum then was added to each of the three tubes and the packed, sedimented cells were resuspended completely.

II. *Method of Testing the Sensitized Rh-Positive Cells for Agglutination With Anti-Rh Sera.*—To 0.05 c.c. of each of the three sensitized cell suspensions pipetted into three rows of three small tubes each were added in Row (1), 0.05 c.c. undiluted anti-D (Rh_0) serum containing Rh agglutinins of the complete (saline) variety; in Row (2), 0.05 c.c. undiluted anti-D (Rh_0) serum containing Rh agglutinins of the incomplete (albumin) variety; and in Row (3), 0.05 c.c. of undiluted, normal, adult, human serum. After standing for one hour at room temperature, the tubes were centrifuged for two minutes at 1,500 revolutions per minute and read macroscopically for agglutination. The results are seen in Table II.

TABLE II. AGGLUTINATION BY ANTI-RH SERA OF RH-POSITIVE CELLS TREATED WITH THE "SUPERNATANT FRACTIONS" OF SERUM (REE), SERUM (FIS), AND A NORMAL SERUM

	SUPERNATANT FRACTIONS		
	A SERUM (REE)	B SERUM (FIS)	C NORMAL SERUM
1. Anti-D (Rh_0) agglutinins (complete)	±	++++	++++
2. Anti-D (Rh_0) agglutinins (incomplete)	–	++++	++++
3. Normal, adult, human serum	–	±	–

All dilutions made with undiluted, normal, adult, human serum of Group O.

The "supernatant fraction" of serum (Ree) completely suppresses agglutination of the test cells. In contrast, the "supernatant fraction" of a serum containing Rh antibodies of the incomplete (albumin) variety fails to inhibit the agglutination of Rh-positive cells upon the subsequent addition of Rh agglutinins of either the complete (saline) or the incomplete (albumin) variety, behaving exactly as the "supernatant fraction" of the normal serum.

Attention should be called to the fact that Rh-positive cells treated with a "supernatant fraction" containing incomplete (albumin) Rh antibodies were only slightly agglutinated, if at all, following their resuspension in undiluted, normal, adult, human serum (3B). This observation suggests that the incomplete (albumin) type of Rh agglutinins can be removed from Rh-positive cells by simple washing, a finding which will be discussed later in detail.

As mentioned, the supernatants obtained in step 4 during the preparation of the preceding experiment were kept for further study. They are nothing else but the absorbed "supernatant fractions" of the Rh antisera used in Experiment II. It was of interest to find out in what way the Rh agglutinin content of these sera was affected by treatment with Rh-positive cells.

Experiment III shows the influence of absorption upon the Rh antibody content of the "supernatant fraction" of the two anti-Rh sera. The titrations were set up in two parts as follows:

Decreasing amounts of the "supernatant fractions" of sera (Ree) and (Fis) respectively, volume 0.05 c.c., in Part I, before absorption, and in Part II, after absorption, were mixed each with 0.05 c.c. of a 2 per cent suspension of heterozygous Rh_1 (CDe/ce) Group O cells. The tubes were shaken thoroughly and allowed to stand for forty-five minutes at room temperature. They then were centrifuged and read macroscopically for agglutination as recorded in Table III. As a diluent for all the dilutions and the test cell suspension, undiluted, normal, adult, human serum of Group O was used.

TABLE III. AGGLUTINATION OF RH-POSITIVE CELLS BY THE "SUPERNATANT FRACTIONS" OF SERUM (REE) AND SERUM (FIS) BEFORE AND AFTER ABSORPTION

	PART I BEFORE ABSORPTION		PART II AFTER ABSORPTION	
SUPERNATANT FRACTION	A SERUM (REE)	B SERUM (FIS)	C SERUM (REE)	D SERUM (FIS)
1. Undiluted	−	++++	−	+
2. 1:2	−	++++	−	±
3. 1:4	+	++++	+	±
4. 1:8	+	+++	++	−
5. 1:16	++	++	++++	−
6. 1:32	++	+	++++	−
7. 1:64	+++	±	++++	−
8. 1:128	+++	−	++++	−
9. 1:256	+++	−	++++	−
10. 1:512	++	−	++++	−
11. 1:1,024	++	−	+++	−
12. 0	−	−	−	−

All dilutions made with undiluted, normal, adult, human serum of Group O.

The comparison of the "supernatant fractions" of serum (Ree) and serum (Fis) respectively reveals an interesting contrast. Treatment of the "supernatant fraction" of serum (Ree) with Rh-positive cells has resulted in a surprising increase in the degree of agglutination obtained rather than in a decrease as would be expected. On the other hand, treatment of the "supernatant fraction" of serum (Fis), carried out under identical conditions, has resulted in a considerable loss of the Rh antibody content. Apparently a blocking type of Rh antibody has been absorbed from the "supernatant fraction" of serum (Ree), allowing the incomplete (albumin) type of Rh antibody, also present, to act on the Rh-positive test cells to a greater degree than before absorption. This paradoxical phenomenon seems to be explained best by the assumption of differences in the avidity of the various types of Rh antibodies for Rh-positive cells.

That absorption of the "supernatant fraction" of serum (Ree) with Rh-positive cells indeed increases rather than decreases its agglutinating potency under certain quantitative conditions is shown in the following Experiment IV.

I. *Method of Absorption of Supernatant Fraction of Serum (Ree)*.*—

1. 1.0 c.c. of the undiluted "supernatant fraction" of serum (Ree) was added to 1.0 c.c. of thrice washed, packed heterozygous Rh_1 (CDe/ce) Group O cells, mixed and kept for thirty minutes in the refrigerator at 4° C.
2. Following centrifugation for ten minutes at 4,500 revolutions per minute, the supernatant fluid was aspirated, saved, and labelled "Absorbed Fluid No. 1." An aliquot was removed for testing purposes.
3. The remaining "Absorbed Fluid No. 1" was reabsorbed with an equal volume of washed, packed cells, kept at 4° C., and centrifuged; the supernatant was aspirated and labelled "Absorbed Fluid No. 2."

II. *Quantitative Testing of the Absorbed Supernatant Fraction of Serum (Ree).*—Decreasing amounts of (A) the "supernatant fraction" of serum (Ree) before absorption, (B) after the first absorption ("Absorbed Fluid No. 1"), (C) after the second absorption ("Absorbed Fluid No. 2"), volume 0.05 c.c., were mixed with 0.05 c.c. of a 2 per cent suspension of heterozygous Rh_1 (CDe/ce) Group O cells. Undiluted, human serum was used as a

*For this experiment the lyophilized "supernatant fraction" of serum (Ree) was dissolved in undiluted, normal, adult, human serum of Group A instead of in 0.9 per cent saline solution.

diluent throughout the experiment. After standing for forty-five minutes at room temperature the tubes were centrifuged at 1,500 revolutions per minute for two minutes. The resulting agglutination as read macroscopically is recorded in Table IV.

TABLE IV. AGGLUTINATION OF RH-POSITIVE CELLS BY THE "SUPERNATANT FRACTION" OF SERUM (REE) AND THE ABSORBED FLUIDS PREPARED FROM THIS FRACTION

SUPERNATANT FRACTION OF SERUM (REE)	A UNTREATED	B ABSORBED FLUID NO. 1	C ABSORBED FLUID NO. 2
1. Undiluted	−	±	++
2. 1:2	−	+	++
3. 1:4	+	++	+
4. 1:8	+	++	+
5. 1:16	++	+++	++
6. 1:32	++	+++	+++
7. 1:64	++	++++	++
8. 1:128	++	++++	++
9. 1:256	+++	++++	+
10. 1:512	++	++++	±
11. 1:1,024	++	+++	−
12. 1:2,048	±	++	−
13. 1:4,096	±	+	−
14. 1:8,192	−	±	−
15. 1:16,384	−	±	−
16. 1:32,768	−	−	−
17. 1:65,536	−	−	−
18. 0	−	−	−

All dilutions made with undiluted, normal, adult, human serum of Group O.

Treatment of the "supernatant fraction" of serum (Ree) with an equal volume of packed Rh-positive cells increases the degree of agglutination of Rh-positive cells produced by this fraction. It also reduces the prozone. Upon further absorption, however, a decrease in agglutination of Rh-positive cells occurs, suggesting that following the removal of the blocking variety of Rh agglutinins, the incomplete (albumin) variety of Rh agglutinins is removed next.

In order to study the specificity of the blocking effect of the "supernatant fraction" of serum (Ree) upon the agglutination of Rh-positive cells, the following experiment was set up:

A 10 per cent suspension of homozygous Rh_1 (CDe/Ce) Group O cells was mixed with equal volumes of (1) undiluted, (2) 1:10 diluted, and (3) 1:100 diluted "supernatant fractions" respectively of (A) anti-Rh serum (Ree), and (B) a normal, adult, human serum. The washing and resuspending procedures were identical with those described in detail under Experiment I.

The blood cells treated in this way were tested for agglutination in the following manner:

To 0.05 c.c. of each of the sensitized cell suspensions was added in Part I, 0.05 c.c. of an undiluted anti-D (Rh_0) serum, and in Part II, 0.05 c.c. of an undiluted anti-C (Rh') serum. Both anti-Rh sera contained Rh antibodies of the complete (saline) variety. The tubes were allowed to remain thirty minutes at room temperature, then were centrifuged and read macroscopically for agglutination as shown in Table V.

Homozygous Rh_1 (CDe/Ce) cells treated with the "supernatant fraction" of serum (Ree) fail to be agglutinated upon the addition of anti-D (Rh_0) serum. However, the addition of anti-C (Rh'-70%) serum brings about agglutination without showing any trace of inhibition.

TABLE V. AGGLUTINATION BY ANTI-D (Rh_0) AND ANTI-C (Rh') SERA OF RH-POSITIVE CELLS TREATED WITH THE "SUPERNATANT FRACTION" OF SERUM (REE)

SUPERNATANT FRACTIONS	PART I ANTI-D (Rh_0) SERUM		PART II ANTI-C (Rh') SERUM	
	A SERUM (REE)	B NORMAL SERUM	A SERUM (REE)	B NORMAL SERUM
1. Undiluted	–	++++	++++	++++
2. 1:10	–	++++	++++	++++
3. 1:100	+	++++	++++	++++

All dilutions made with undiluted, normal, adult, human serum of Group O.

A similar type of experiment carried out with Rh_2 (cDE/e) cells using an anti-E (Rh") serum, proved that agglutination of Rh_2 cells by an anti-E antibody was not inhibited by the "supernatant fraction" of serum (Ree). The blocking effect of the "supernatant fraction" of serum (Ree), therefore, corresponds to the specificity of an anti-D (Rh_0) antibody. These results parallel the findings obtained by Hattersley and Fawcett[4] in their study of the prozone phenomena shown by several anti-Rh sera.

DISCUSSION

The experiments described in this paper were designed to analyze the nature of the prozone phenomenon of the "supernatant fraction" obtained by dialysis from a certain anti-Rh serum (Ree). The question presented itself as to whether this prozone was caused by a surplus of Rh antibodies or was due to the presence of a new and different type of Rh antibody which inhibited rather than produced agglutination. Rh-positive cells after being exposed to the "supernatant fraction" of serum (Ree) in concentrations which gave the prozone effect should have become agglutinated following removal of an excess of Rh antibodies by multiple washings. Rh-positive cells treated in this manner, however, did not only fail to become agglutinated when resuspended in saline solution or undiluted, normal, adult, human serum, but also could not be clumped any more by the addition of Rh antisera containing Rh antibodies of either the complete (saline) or the incomplete (albumin) varieties. It is difficult to avoid the conclusion that the prozone phenomenon under discussion must be caused by an Rh antibody of blocking character rather than by the presence of a surplus of Rh antibodies of the two known varieties (complete and incomplete).

Furthermore, maximum agglutination of Rh-positive cells (4-plus agglutination) should have occurred upon serial dilution of the "supernatant fraction" of serum (Ree) provided the prozone phenomenon was due only to an excess of Rh antibodies and not to the presence of a particular blocking antibody. This fraction, however, never produced agglutination of the 4-plus strength, yet 4-plus agglutination occurred following its absorption with Rh-positive cells. The new type of Rh antibody herein described must, therefore, possess a greater avidity for the Rh antigen of the red blood cell than the incomplete (albumin) Rh antibody. This latter antibody in turn, as evidenced by the experiments of Coombs and Race[5] and Levine and Waller,[6] has a greater avidity

for the Rh antigen than the complete (saline) Rh antibody. In other words, there is a difference in the speed of combination between the various types of Rh antibodies and the Rh antigen.

In addition to differences in the speed of union between Rh antigen and the different Rh antibodies, there seems also to be a difference in the strength of the Rh antigen-antibody combinations. Multiple washings with saline solution removed the incomplete (albumin) variety of Rh antibody from Rh-positive cells in contrast to the new type of Rh antibody which remained attached to the cells, apparently not being influenced by the multiple washing procedure.

CONCLUSIONS

1. The prozone phenomenon observed in the "supernatant fraction" obtained by dialysis from an anti-Rh serum (Ree) was found to be due to a new (third) type of Rh antibody rather than to a surplus of Rh antibodies of known varieties.

2. This new type of Rh antibody when mixed with Rh-positive cells prevented their agglutination upon the subsequent addition of Rh antisera containing Rh agglutinins of both the complete (saline) and the incomplete (albumin) variety.

3. In contrast, the "supernatant fractions" obtained by dialysis from sera containing the incomplete (albumin) variety of Rh antibodies, as well as from normal sera, failed to reveal any inhibitory effect on the agglutination of Rh-positive cells.

4. The third type of Rh antibody could not be removed from Rh-positive cells by multiple washings with saline solution, in contrast to the incomplete (albumin) type of Rh antibody which can be eluted by a series of washings.

5. Absorption of the "supernatant fraction" of serum (Ree) with equal volumes of Rh-positive cells increased rather than decreased the agglutinating properties of this fraction. In contrast, the agglutinin titer of a "supernatant fraction" containing only the incomplete (albumin) type of Rh antibody was reduced materially by this treatment.

6. The third Rh antibody described was found to have anti-D (Rh_o) specificity.

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STUDIES ON Rh ANTIBODIES

III. Analysis of a Zone Phenomenon in an Rh Antiserum Split by Dialysis Into Four Fractions

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THE observations reported in the two preceding papers[1, 2] dealt with the anti-Rh serum (Ree) and its two fractions obtained by dialysis for eighteen hours following the method described previously.[3] However, the amount of precipitate which formed during this period varied for different sera. It was influenced by the character of the serum itself, such as its lipoid content, and depended, to a certain extent at least, upon the amount of distilled water used, the number of changes of distilled water made during each dialysis period, and other factors. Furthermore, after removal of the first precipitate which formed after eighteen hours, additional precipitation occurred following continued dialysis. Assuming that precipitates formed during various stages of dialysis would also correspond to different globulin fractions of the serum, their antibody content was examined. The method used constituted a further division of the original serum into four fractions (three "globulin fractions" and one "supernatant fraction") in contrast to the previous dialysis technique which allowed the splitting of anti-Rh sera into two fractions only (the "globulin fraction" and the "supernatant fraction"). This more refined method of dialysis of anti-Rh sera revealed results which are the subject of this report.

TECHNIQUE

All of the dialysis experiments reported in this and in the previous communications were performed using the same type of dialysis membrane, which was prepared by cutting a sufficient length of viscose tubing,* using 27/32 inch casing of a size approximately 43 mm. in diameter. A bag was made from the piece of tubing by folding one end upon itself several times and securing it tightly with twine. The tubing was opened by moistening it with distilled water, and was filled with distilled water, testing for leakage by placing the contents of the bag under as great a tension as possible. The respective serum specimen to be examined, which was stored frozen at –30° C., was thawed at room temperature. Using a small glass funnel, 100 c.c. of this serum were poured into the viscose tubing bag. The air was forced out of the membrane without appreciable loss of the serum and the open end of the bag was twisted tightly in order to put the serum under tension. This end then was secured firmly with twine. After weighting the bottom end of the dialyzing bag with a one-hole rubber stopper, the outside was rinsed thoroughly with distilled water. The bag then was suspended from a glass stirring rod into a battery jar holding 12,000 c.c. of freshly distilled water precooled to 4° C. by storage in the refrigerator for at least four hours prior to dialysis. Dialysis was allowed to continue in this first battery jar in the refrigerator at 4° C. for six hours. After this period the dialyzing bag was transferred to a second battery jar also containing

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*The viscose tubing consists of regenerated cellulose and is made commercially by the Visking Corporation, Chicago, Ill. It is obtainable from laboratory supply houses such as the Fisher Scientific Company, Pittsburgh, Pa., or the Will Corporation, Rochester, N. Y.

12,000 c.c. of freshly distilled water precooled to 4° C., and dialysis was permitted to continue in the refrigerator for twelve hours. The total time period allotted for this first phase of dialysis was eighteen hours.

The bag then was inverted several times, resuspending completely the precipitate which had settled to the bottom, and the exterior of the membrane was rinsed with distilled water. The bottom of the bag was opened by severing the string and the contents were emptied into a large glass beaker.

Approximately half of the dialysate was transferred to a plastic centrifuge tube of 80 c.c. capacity and centrifuged in an angle centrifuge at 5,000 revolutions per minute for thirty minutes. The supernatant was decanted off and saved. The remaining half of the dialysate was added to the same centrifuge tube containing the sediment obtained in the previous run and spun down, thus collecting all of the precipitate in the one centrifuge tube. The supernatants were combined as "supernatant fraction" and saved for further dialysis. The tube containing the packed sediment was inverted on coarse filter paper and allowed to drain for thirty minutes at room temperature.

The precipitate recovered from the 100 c.c. of serum was dissolved in 10 c.c. of 0.9 per cent saline solution which was added in divided amounts. After each portion of saline solution was added, the precipitate was stirred vigorously with a glass stirring rod. Final solution occurred readily at room temperature within fifteen to thirty minutes. The globulin solution was transferred to a double-thickness, Pyrex, conical centrifuge tube and spun down in an angle centrifuge for fifteen minutes at 1,500 revolutions per minute. Only a slight amount of insoluble material remained and this was discarded. The clear solution was lyophilized in 1 c.c. amounts and stored at room temperature. This material is referred to as "globulin fraction No. 1," which represents a ten times concentration as compared with the native serum.

The "supernatant fraction" obtained from this first phase of dialysis was again subjected to eighteen hours of dialysis exactly as described for the first period. The precipitate which formed was collected in the same manner as stated for "globulin fraction No. 1." Inasmuch as it was slightly less in amount than the first precipitate, it was dissolved in only 5 c.c. of 0.9 per cent saline solution. The resulting solution was labelled "globulin fraction No. 2," constituting a twenty times concentration as compared with the native serum.

The "supernatant fraction" recovered following the second stage of dialysis was dialyzed a third time over a period of eighteen hours as already described in detail. The small amount of precipitate obtained was dissolved in 2 c.c. of physiologic saline solution. This was labelled "globulin fraction No. 3" and amounts to a fifty times concentration as compared with the native serum.

The final supernatant fluid recovered from the third dialysis was lyophilized in 10 c.c. amounts and is referred to as the "supernatant fraction." When employed for experimental purposes the dried "globulin fractions" were dissolved in distilled water and the salt-free "supernatant fraction" in 0.9 per cent saline solution in order to maintain isotonicity in all fractions.

EXPERIMENTAL

The Rh antibody content of the four fractions derived from serum (Rec) was determined by means of quantitative titrations in an experiment which was carried out in two parts. In Part I, all dilutions were made in physiologic saline solution, while in Part II, undiluted, normal, adult, human serum was used as a diluent. The experiment itself was performed as follows:

Decreasing amounts of (A) native serum, (B) "globulin fraction No. 1," (C) "globulin fraction No. 2," (D) "globulin fraction No. 3," (E) "supernatant fraction," volume 0.05 c.c., each were mixed with 0.05 c.c. of a 2 per cent suspension of washed Rh_2 (cDE/c) Group O cells. The tubes were shaken well, kept for one hour at room temperature and centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutination is shown in Table I.

TABLE I. AGGLUTINATION OF RH-POSITIVE GROUP O CELLS BY SERUM (REE) AND ITS FOUR FRACTIONS

DILUENT	PART I 0.9% SALINE					PART II UNDILUTED HUMAN SERUM				
SERUM (REE)	A NATIVE SERUM	B GLOBULIN FRACTION NO. 1	C GLOBULIN FRACTION NO. 2	D GLOBULIN FRACTION NO. 3	E SUPERNATANT FRACTION	A NATIVE SERUM	B GLOBULIN FRACTION NO. 1	C GLOBULIN FRACTION NO. 2	D GLOBULIN FRACTION NO. 3	E SUPERNATANT FRACTION
1. Undiluted	+	++++	–	–	–	+++	++++	++++	++++	–
2. 1:2	±	++++	–	–	–	++++	++++	+++	++++	–
3. 1:4	–	++++	–	–	–	++++	++++	++	++++	–
4. 1:8	–	++++	–	–	–	+++	++++	+	++++	±
5. 1:16	–	+++	–	–	–	++	+++	+	++++	±
6. 1:32	–	+++	–	–	–	+	+++	±	+++	+
7. 1:64	–	++	–	–	–	±	++	–	++	+
8. 1:128	–	+	–	–	–	+	+	–	+	++
9. 1:256	–	–	–	–	–	++	+	–	±	+
10. 1:512	–	–	–	–	–	++	±	–	–	±
11. 1:1,024	–	–	–	–	–	++	–	–	–	–
12. 1:2,048	–	–	–	–	–	++	–	–	–	–
13. 0	–	–	–	–	–	–	–	–	–	–

–, No gglutination.
±, Faint agglutination.
+, Slight agglutination.
++, Marked agglutination.
+++, Strong agglutination.
++++, Very strong agglutination.

"Globulin fraction No. 1" contains a potent complete (saline) Rh agglutinin. "Globulin fractions No. 2 and No. 3" as well as the "supernatant fraction" are devoid of any complete (saline) Rh antibodies. It should be kept in mind that the "globulin fractions" compared with the native serum are 10, 20, and 50 times concentrated respectively. When undiluted, human serum is used as a diluent, the native serum reveals the presence of Rh agglutinins, again exhibiting a definite zone phenomenon in the middle of the series of dilutions. "Globulin fractions No. 2 and No. 3" contain Rh antibodies of the incomplete (albumin) variety, decreasing in strength in a straight line upon dilution, indicating that it is possible to separate the complete and the incomplete Rh antibodies by dialysis. Obviously, therefore, prolonged dialysis of the serum has resulted finally in the precipitation of "globulin fractions" containing the incomplete (albumin) Rh antibodies also.

This experiment might well be compared with the Experiment I described in the first paper of this series.[1] The single eighteen-hour dialysis did not bring about the precipitation of the incomplete (albumin) Rh agglutinins which remained in solution. Nevertheless, even after three eighteen-hour dialysis periods, a certain amount of incomplete (albumin) Rh antibodies still remains in the "supernatant fraction" which exhibits a strong prozone phenomenon.

The question arose whether the "supernatant fraction" remaining after dialysis of serum (Ree) for three days would still contain the blocking antibody described in the preceding communication.[2] In order to clarify this point the following experiment was carried out:

I. *Method of Sensitization of Rh-Positive Cells.*—

1. 0.5 c.c. of a 10 per cent suspension of three times washed Rh_2 (cDE/c) Group O cells was added to four rows of six tubes each.
2. Increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, (5) 1:81, and (6) 1:243 of the following materials were added in a volume of 0.5 c.c. per tube as follows:
   - Row (A): Native (untreated) serum (Ree).
   - Row (B): "Supernatant fraction" of serum (Ree).
   - Row (C): Normal adult serum.
   - Row (D): "Supernatant fraction" of a normal adult serum.
3. After shaking the tubes they were kept for thirty minutes in the refrigerator at 4° C.
4. The tubes were centrifuged for ten minutes at 4,500 revolutions per minute and the supernatants were aspirated and discarded.
5. The sediments, consisting of packed red blood cells, were washed thoroughly three times, using 5 c.c. of ice-cold 0.9 per cent saline solution per tube.
6. Following the third washing, all of the saline solution was removed by aspiration.
7. 0.5 c.c. of undiluted, normal, adult, human serum was added to each tube and the cells were resuspended completely in the serum.

II. *Method of Testing the Sensitized Rh-Positive Cells for Agglutination With Anti-Rh Sera.*—The experiment was set up in triplicate. To 0.05 c.c. of the cell suspensions of each of the tubes of rows (A) to (D), pipetted into a new series of small tubes, was added in Part I, 0.05 c.c. of undiluted anti-D (Rh_0) serum containing Rh agglutinins of the complete (saline) variety; in Part II, 0.05 c.c. of undiluted anti-D (Rh_0) serum containing Rh agglutinins of the incomplete (albumin) variety; and in Part III, 0.05 c.c. of undiluted, normal, adult, human serum. After standing for forty-five minutes at room temperature the tubes were centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutinations are seen in Table II.

TABLE II. AGGLUTINATION BY ANTI-RH SERA OF RH-POSITIVE GROUP O CELLS PREVIOUSLY TREATED WITH NATIVE SERUM (REE) AND ITS "SUPERNATANT FRACTION"

ANTI-RH SERUM	PART I ANTI-D (RH_0) SERUM—COMPLETE VARIETY				PART II ANTI-D (RH_0) SERUM—INCOMPLETE VARIETY				PART III UNDILUTED NORMAL ADULT SERUM (CONTROL)			
	A	B	C	D	A	B	C	D	A	B	C	D
SENSITIZING SERUM	SERUM	SUPERNATANT	SERUM	SUPERNATANT	SERUM	SUPERNATANT	SERUM	SUPERNATANT	SERUM	SUPERNATANT	SERUM	SUPERNATANT
	(REE)		NORMAL		(REE)		NORMAL		(REE)		NORMAL	
1. Undiluted	+	−	++++	++++	++++	−	++++	++++	+	−	−	−
2. 1:3	±	−	++++	++++	++++	±	++++	++++	±	−	−	−
3. 1:9	−	−	++++	++++	++++	+	++++	++++	−	−	−	−
4. 1:27	−	+	++++	++++	++++	++	++++	++++	−	−	−	−
5. 1:81	+	++	++++	++++	++++	++++	++++	++++	−	−	−	−
6. 1:243	++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−

All dilutions made with undiluted, normal, adult, human serum of Group O.

As can be seen from Experiment II, blocking antibodies are present in the "supernatant fraction" of serum (Ree). The inhibitory effect of the "supernatant fraction" manifests itself against both the complete (IB) and the incomplete (IIB) varieties of anti-D (Rh_0) antibodies, though stronger against the complete (saline) Rh agglutinins. The native serum (Ree), too, inhibits the complete (saline) anti-Rh agglutinins (IA). Strangely enough, however, it fails to inhibit the agglutination of Rh-positive cells by the incomplete (albumin) variety of anti-Rh agglutinins (IIA). The failure of Rh-positive cells previously sensitized by the native serum (Ree) to be agglutinated when taken up in undiluted, normal, adult, human serum (IIIA)* confirms the observations made in the preceding paper[2] that the incomplete (albumin) variety of Rh antibody can be removed from sensitized cells, to a great extent at least, by multiple washings.

An attempt now was made to demonstrate in the native serum proper the presence of blocking antibodies against the incomplete (albumin) Rh antibody by subjecting Rh-positive cells sensitized by the native serum to a more thorough washing procedure than that used in the preceding experiment. To this end the following experiment was carried out:

I. *Method of Sensitization of Rh-Positive Cells.*—

1. 0.1 c.c. of a 10 per cent suspension of three times washed heterozygous Rh_2 (cDE/c) Group O cells was added to eight rows consisting of ten tubes each.
2. Increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, (5) 1:81, (6) 1:243, (7) 1:729, (8) 1:2,187, (9) 1:6,561 and (10) 1:19,683 of the following materials were added in a volume of 0.1 c.c. per tube as follows:
   Row (A): Native (untreated) serum (Ree).
   Row (B): "Supernatant fraction" of serum (Ree).
   Row (C): Normal, adult serum.
   Row (D): "Supernatant fraction" of a normal, adult serum.
   The experiment was set up in duplicate.
3. The tubes were shaken and kept for thirty minutes at 4° C.
4. After centrifuging the tubes for ten minutes at 3,000 revolutions per minute the supernatants were aspirated and discarded.
5. The sediments, consisting of packed red blood cells, were washed thoroughly four times using 2 c.c. of ice-cold 0.9 per cent saline solution per tube.
6. After the fourth washing all of the saline solution was removed completely by aspiration.
7. 0.1 c.c. of undiluted, normal, adult, human serum was added to each tube and the cells were resuspended in the serum.

II. *Method of Testing the Sensitized Rh-Positive Cells for Agglutination with Anti-Rh Sera.*—In Part I, 0.1 c.c. of 1:3 diluted anti-D (Rh_0) serum containing Rh agglutinins of the incomplete (albumin) variety was added to each of the tubes, and in Part II, 0.1 c.c. of undiluted, normal, adult, human serum was added to each of the tubes. The tubes were allowed to stand at room temperature for one hour and were centrifuged for two minutes at 3,000 revolutions per minute. The agglutination as read macroscopically is recorded in Table III.

*Weak agglutination obtained in the first two tubes in Row (A) of Part I can be explained by the presence of traces of Rh antibodies not yet completely removed by washing as indicated by an identical reaction in the first two tubes of Row (A) of Part III, in which undiluted, normal, adult, human serum was used as a control in place of anti-Rh serum.

TABLE III. AGGLUTINATION BY AN ANTI-RH SERUM OF THE INCOMPLETE VARIETY OF RH-POSITIVE GROUP O CELLS PREVIOUSLY TREATED WITH NATIVE SERUM (REE) AND ITS "SUPERNATANT FRACTION"

ANTI-RH SERUM	PART I ANTI-D (Rh_0) SERUM—INCOMPLETE VARIETY				PART II UNDILUTED, NORMAL, ADULT SERUM (CONTROL)			
SENSITIZING SERUM	A SERUM (REE)	B SUPERNATANT (REE)	C SERUM NORMAL	D SUPERNATANT NORMAL	A SERUM (REE)	B SUPERNATANT (REE)	C SERUM NORMAL	D SUPERNATANT NORMAL
1. Undiluted	+	−	+++	+++	+	−	−	−
2. 1:3	±	−	+++	+++	±	−	−	−
3. 1:9	−	−	+++	+++	−	−	−	−
4. 1:27	−	±	+++	+++	−	−	−	−
5. 1:81	−	+	+++	+++	−	−	−	−
6. 1:243	±	++	+++	+++	−	−	−	−
7. 1:729	+	+++	+++	+++	−	−	−	−
8. 1:2,187	+++	+++	+++	+++	−	−	−	−
9. 1:6,561	+++	+++	+++	+++	−	−	−	−
10. 1:19,683	+++	+++	+++	+++	−	−	−	−

All dilutions made with undiluted, normal, adult, human serum of Group O.

Experiment III shows that blocking antibodies against the incomplete (albumin) Rh antibody can be demonstrated even in the native serum (Ree). Their demonstration is made possible only by thoroughly washing the sensitized Rh-positive cells, a procedure which apparently is necessary to remove Rh antibodies other than the blocking variety which remains attached to the cells in spite of the washing.

Coombs, Mourant, and Race[4-6] have described an important technique which permits the detection of sensitization of Rh-positive cells by Rh antibodies. Rh-positive cells sensitized by Rh antibodies without showing visible agglutination are washed several times and then agglutinated by the addition of an antihuman serum rabbit serum. It was of interest to find out which position the Coombs test would assume in the detection of Rh antibodies as found in the various fractions of the Rh antiserum (Ree). The following experiment was carried out to elucidate this problem.

I. *Method of Sensitization of Rh-Positive Cells.*—

1. 0.1 c.c. of a 10 per cent suspension of washed heterozygous Rh_1 (CDe/ce) cells belonging to blood Group O was added to each of twelve tubes in six rows.
2. Increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, (5) 1:81, (6) 1:243, (7) 1:729, (8) 1:2,187, (9) 1:6,561, (10) 1:19,683, (11) 1:59,049, and (12) 1:177,147 of the following materials were added in a volume of 0.1 c.c. per tube as follows:

   Row (A): Native (untreated) serum (Ree).
   Row (B): "Globulin fraction No. 3" of serum (Ree).
   Row (C): "Supernatant fraction" of serum (Ree).

   These titrations were performed in duplicate.
3. The tubes were shaken and kept for thirty minutes in the refrigerator at 4° C.
4. Following centrifugation for fifteen minutes at 3,000 revolutions per minute, the supernatants were aspirated from the tubes and discarded.
5. The packed red blood cells in each tube were washed three times using 2 c.c. of ice-cold 0.9 per cent saline solution per tube.

6. After the third washing all of the saline solution was removed as completely as possible by aspiration.
7. In Part I, 0.1 c.c. of 0.9 per cent saline solution was added to each of the tubes, while in Part II, 0.1 c.c. of undiluted, normal, adult, human serum was added to each of the tubes and the cells were resuspended in the respective diluents.

II. *Method of Testing the Sensitized Rh-Positive Cells for Agglutination With Antihuman Serum Rabbit Serum.*—In Part I, 0.1 c.c. of 1:30 diluted antihuman serum rabbit serum was added to each of the tubes, and in Part II, an additional 0.1 c.c. of the same undiluted, normal, adult, human serum used for resuspending the cells was added to each of the tubes (control). The tubes were allowed to stand at room temperature for one hour and then were centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutinations are seen in Table IV.

TABLE IV. COOMBS TEST

AGGLUTINATION BY ANTIHUMAN SERUM RABBIT SERUM OF RH-POSITIVE CELLS SENSITIZED WITH NATIVE SERUM (REE) AND TWO OF ITS FRACTIONS RESPECTIVELY

ANTISERA	PART I ANTIHUMAN SERUM RABBIT SERUM			PART II UNDILUTED, NORMAL, ADULT SERUM (CONTROL)		
ANTI-RH SERUM (REE)	A NATIVE SERUM	B GLOBULIN FRACTION NO. 3	C SUPERNATANT FRACTION	A NATIVE SERUM	B GLOBULIN FRACTION NO. 3	C SUPERNATANT FRACTION
1. Undiluted	++++	++	++++	–	–	–
2. 1:3	++++	++++	++++	–	–	–
3. 1:9	++++	++++	++++	–	–	–
4. 1:27	++++	++++	++++	–	–	–
5. 1:81	++++	++++	++++	–	–	–
6. 1:243	++++	++++	++	–	–	–
7. 1:729	++++	++++	+	–	–	–
8. 1:2,187	++++	++++	–	–	–	–
9. 1:6,561	+++	++++	–	–	–	–
10. 1:19,683	+++	++++	–	–	–	–
11. 1:59,049	++	++	–	–	–	–
12. 1:177,147	+	++	–	–	–	–

The exceeding sensitivity of the Coombs test is evident from this experiment. It allows the detection of the sensitization of Rh-positive cells by the native serum (Ree) up to the final dilution used (up to 1:177,000). The "supernatant fraction" of the serum (Ree) containing blocking Rh antibodies gives a positive Coombs test up to a dilution of 1:729, roughly corresponding to its content of blocking Rh antibody (see Experiment III). The strength of the Coombs test obtained with "globulin fraction No. 3" is surprising. This "globulin fraction" contained Rh antibodies of the incomplete (albumin) variety demonstrable up to dilutions not higher than 1:256 (see Experiment I) and yet the Coombs test is still definitely positive with the highest dilution used in this experiment (1:177,000). This finding is even more surprising if one considers the results of Part II of this experiment. Rh-positive test cells treated in an exactly identical way as in Part I were resuspended in undiluted, adult serum without showing any trace of agglutination, pointing to the fact that the incomplete (albumin) Rh antibodies originally present were washed away or at least rendered ineffective by the washing procedure. It should also be mentioned that in experiments not reported in this paper, "globulin fraction No. 3" was found to be devoid completely of Rh blocking antibodies.

DISCUSSION

Dialysis has been used during the last three years in this laboratory as a means of splitting anti-Rh sera into two fractions, namely, the precipitate, referred to as the "globulin fraction," and the "supernatant fraction" which was obtained following removal of the precipitate by centrifugation. As previously reported,[3] the precipitate frequently contained the complete (saline) Rh antibody while the incomplete (albumin) Rh antibody was found in the supernatant. In this manner certain anti-Rh sera which were useless as test sera could be made into potent reagents for Rh determinations using the "globulin fraction." This fraction can be concentrated easily, depending upon the volume of saline solution in which it is dissolved. The method also proved that Rh antibodies of different varieties might be separated from each other because they apparently are associated with different protein fractions of serum.

In this paper a more quantitative method was used. Dialysis was continued over a period of three days during which time precipitates formed after each eighteen-hour period were collected separately and examined for their Rh antibody content. Prolonged dialysis finally led to the precipitation of a good portion of the incomplete (albumin) variety of Rh antibody. However, the remaining supernatant fluid still contained an Rh antibody with typical blocking qualities. It seems doubtful whether further dialysis would have resulted also in precipitation of this type of Rh antibody. It should be interesting to find out how the method of fractionated alcohol precipitation, as carried out by Cohn and associates, compares with the relatively simple procedure of dialysis used in the investigations reported. It seems unlikely that dialysis in the cold denatures proteins at all, which might give this method a certain advantage over others.

Differences in avidity among the various types of Rh antibodies manifest themselves upon washing sensitized Rh-positive cells. The incomplete (albumin) variety of Rh antibody can be rather easily washed off with three or four thorough washings as proved by the fact that Rh-positive cells previously sensitized with this type of Rh antibody and then washed failed to be agglutinated when suspended in undiluted, adult, human serum or albumin solution respectively. It was not possible, however, to remove the blocking type of Rh antibody present in the "supernatant fraction" of serum (Ree) even by a series of thorough washings. As a matter of fact, the blocking effect of the native serum (Ree) became apparent only following washing procedures. As one might speculate, the blocking type of Rh antibody "takes over" and readily combines with the Rh antigens which become available following the removal of the incomplete (albumin) Rh antibodies.

The name blocking antibody used in this paper denotes an Rh antibody which not only fails to agglutinate but even interferes with the agglutination of Rh-positive cells when they are suspended in an albumin-containing medium. It should be recalled that Wiener[7] previously called the incomplete (albumin) variety of Rh antibody the "blocking" antibody because it prevented agglutination of Rh-positive cells by the complete (saline) variety of Rh antibody in

physiologic saline solution. However, this blocking effect of the incomplete (albumin) Rh antibody was evident only when physiologic saline solution was used as a diluent, but not in undiluted serum or in albumin solution. The blocking antibody described in this paper inhibits agglutination of Rh-positive cells even in an albumin-containing medium and it truly blocks the incomplete (albumin) variety of Rh antibody as well as the complete (saline) variety.

Therefore, the existence of three varieties of Rh antibodies can be assumed: (1) the complete or saline variety, (2) the incomplete or albumin variety, and (3) the blocking variety of Rh antibody. On the basis of their studies, Hill and Haberman[8, 9] already felt that there were three types of Rh antibodies, namely (1) the classical agglutinin, (2) the "blocking" antibody (as determined by Wiener[7]), and (3) the "eryptagglutinoid" (as determined by the developing test[8, 9] or Coombs test[4, 5]). The experiments reported in this paper, moreover, point to the likelihood that there is a fourth type of Rh antibody for the following reasons. Experiment IV of this paper reveals the rich content of Coombs antibody in "globulin fraction No. 3." This "globulin fraction No. 3" also contained an incomplete (albumin) Rh antibody with a titer of 256. However, following multiple washings of Rh-positive cells sensitized with this "globulin fraction," no agglutination was demonstrable when the washed, sensitized cells were suspended in undiluted, normal, adult serum. Apparently washing had removed the incomplete (albumin) Rh antibody. Nevertheless, the Coombs test carried out with washed Rh-positive cells sensitized with the "globulin fraction No. 3" showed the presence of an Rh antibody with a titer of at least 177,000. This observation strongly suggests, therefore, that we are dealing with a type of Rh antibody which neither agglutinates Rh-positive cells nor exhibits a blocking effect. Still the possibility cannot be overlooked entirely that traces of incomplete (albumin) Rh antibodies, by themselves insufficient in strength to agglutinate the cells but sufficient to give a positive Coombs test, were still present and attached to the Rh-positive cells under investigation. However, the latter explanation is unlikely considering the difference in titer between the incomplete (albumin) Rh antibody on the one hand and the Coombs antibody on the other.

There seems to be a reciprocal relationship between the capacity of the various types of Rh antibody to agglutinate Rh-positive cells and their ability to be removed by washing; that is to say, Rh antibodies which produce visible agglutination of Rh-positive cells can be removed more easily by washing procedures than Rh antibodies that sensitize Rh-positive cells without producing visible agglutination. Considerable differences in the speed and strength of the union between the various Rh antibodies and Rh antigen must exist.

The so-called unitarian conception of antibody functions is adhered to by the majority of immunologists at the present time. The well-known various manifestations of antigen-antibody reactions such as lysis, agglutination, precipitation, and so on, are explained by this concept on the basis of physicochemical differences of the anitgen rather than of the antibody, depending mainly upon the size of the antigen and the medium in which the antigen-antibody reaction occurs. Therefore, the same antibody that might produce

precipitation in one instance could cause agglutination or lysis of cells under different experimental conditions. The investigations reported in this series of communications are dealing with antibody functions of identical specificity directed against one antigen only, namely the D (or Rh_0) antigen present in the same physicochemical state in red blood cells. Yet, antibodies directed against this antigen have been found to be associated with separate protein fractions of the antiserum and can be distinguished from each other by their avidity for this Rh antigen and by their reaction with it under various experimental conditions. It seems difficult to see how the findings reported can conform to the present unitarian concept of antigen-antibody reactions which appears to need some revision or amplification.

Two theories present themselves in connection with the differences in the antibody functions reported. The first theory would postulate the existence of three or four varieties of independent Rh antibodies which are sharply separated from each other both by the nature of their functions as well as by their association with different protein fractions of the serum. In contrast, the second theory might refuse to accept the existence of sharply separated antibody functions and rather hold to the belief in a gradual change of one antibody function into another, assuming the existence of an Rh antibody spectrum of changing activity.

CONCLUSIONS

1. Prolonged dialysis of an anti-Rh serum (Ree) against measured amounts of distilled water for three eighteen-hour periods resulted in the precipitation of globulins which were collected individually at the end of each eighteen-hour period. Three fractions obtained in this way were referred to as "globulin fractions No. 1, No. 2, and No. 3" respectively. The clear supernatant fluid remaining at the end of the third period of dialysis was called the "supernatant fraction."

2. "Globulin fraction No 1" revealed the presence of a complete (saline) anti-Rh agglutinin which was not found in any other fraction.

3. "Globulin fractions No. 2 and No 3" contained an Rh antibody of the incomplete (albumin) variety.

4. The "supernatant fraction," still containing Rh agglutinins of the incomplete (albumin) variety, exhibited a strong prozone phenomenon.

5. The prozone phenomenon of the "supernatant fraction" is attributed to the presence of a peculiar Rh antibody which inhibits agglutination of Rh-positive cells by both the complete (saline) and the incomplete (albumin) variety of Rh antibodies. It acts, therefore, as a true blocking antibody (third variety of Rh antibody).

6. The presence of this Rh blocking antibody also could be demonstrated in the untreated, native anti-Rh serum (Ree) by subjecting Rh-positive cells sensitized with this serum to thorough washing procedures which apparently resulted in the removal of the incomplete (albumin) variety of Rh antibody.

7. Rh-positive cells sensitized with "globulin fraction No. 3" failed to show macroscopically visible agglutination after being washed thoroughly and

suspended in undiluted, normal, adult, human serum. This fraction contained only incomplete (albumin) Rh antibodies of low titer and was devoid of Rh blocking antibodies. Nevertheless, cells sensitized with it were strongly agglutinated following the addition of antihuman serum rabbit serum (Coombs test), suggesting the existence of a fourth variety of Rh antibody.

8. The occurrence in one anti-Rh serum of certainly three and possibly four varieties of Rh antibodies of the same specificity, anti-D (Rh_0), but of different functions is described. They produce at the extremes of the spectrum of their activity either agglutination or blocking of Rh-positive cells with one variety standing in between which causes neither phenomenon, being demonstrable only by means of the Coombs test. The possible significance of these findings in relation to the validity of the present unitarian concept of antibody functions is discussed.

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METABOLISM, TOXICITY, AND MANNER OF ACTION OF GOLD COMPOUNDS IN THE TREATMENT OF ARTHRITIS

VIII. The Effect of BAL and Other Thiol Compounds in Preventing the Inhibition of Oxygen Consumption of Rat Tissues Produced by Gold Salts

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BOTH organic and inorganic gold compounds have been used for many years in the treatment of rheumatoid arthritis. The value of this form of treatment is subject to argument, and final appraisal on the basis of therapeutic trial apparently is difficult or impossible. The use of gold compounds in rheumatoid arthritis remains on an empiric basis, and even with carefully controlled administration, toxic reactions have been a limiting factor in their use. In order to place this form of treatment on a more rational basis, information regarding the manner by which gold salts produce their effects in the body must be available.

An intensive study of the metabolism and toxicity of the various gold compounds used has been made in this laboratory.[1-6] It was found that when gold compounds are injected intramuscularly into white rats, the gold is deposited in various tissues of the body, primarily in the liver and kidney.[2] The amount and site of deposition seem to depend on the physical properties of the compound studied. Also, the severity of the histopathology in gold-treated animals is roughly proportional to the quantity of gold laid down in the tissue.

We became interested in whether the respiration of these tissues, as measured by oxygen consumption, is influenced by the presence of gold and whether a correlation exists between the concentration of gold in the tissue and its rate of respiration. If such a relationship between deposition of gold and activity of the enzyme systems in these tissues exists, it may provide an insight into the mechanism of gold toxicity. Using the Warburg technique, it was found that the oxygen consumption of rat kidney and liver slices was inhibited in vitro by the inorganic compounds, gold chloride and gold sodium thiouslfate. The organic compounds, sodium succinimido-aurate, gold sodium thiomalate, and gold thioglucose did not cause inhibition.[6]

When 2, 3-dimercaptopropanol (BAL), developed during the war as an effective antidote against acute arsenical poisoning, became available, it was shown to be effective also against other heavy metals, cadmium, mercury, zinc, and copper.[7-14] Furthermore, recent reports indicate the apparent clinical value of BAL in treating gold toxicity in human beings and suggest that its administration results in an augmented urinary excretion of gold under these conditions.[15-17]

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It is now generally accepted that the toxicity of heavy metals, such as arsenic, is due to the inactivation of the sulfhydryl groups in enzyme proteins of the tissues. The action of the thiols is attributed to their effective competition for the heavy metal with the sulfhydryl groups in various body proteins. Arsenic and gold are closely related chemically and it is reasonable to assume that their biochemical reactions are similar. It is suggested that the toxicity of gold is due to its combination with sulfhydryl groups in enzyme proteins, and the sulfhydryl (thiol) groups in BAL compete effectively for the gold combined in the tissue protein, thus restoring the normal enzyme system.

This study is concerned with the effect of BAL and other thiol compounds on the respiration of tissues in vitro, and their possible effect in preventing inhibition of oxygen consumption produced by gold compounds. Obviously, only those gold compounds observed to produce such inhibition in previous studies could be investigated.

METHODS

Measurements of oxygen consumption were made with the usual type of Warburg constant volume manometer. The carbon dioxide produced was absorbed by 0.3 c.c. of 20 per cent potassium hydroxide solution contained in the central well on pleated filter paper. After introduction of the tissue slice, the manometer flasks were immersed in a water bath at 38° C., flushed with pure warm oxygen (38° C.) for ten minutes, and shaken at a rate of 110 oscillations per minute. The suspending medium was a phosphate-buffered physiologic salt solution (pH 7.4) containing 0.2 per cent glucose as described by Krebs.[18] The total volume of fluid in each flask was 2.5 cubic centimeters.

The tissues were taken from healthy male and female white rats which had been fed a standard stock diet. The animals were killed by a blow on the head and the kidneys and/or liver were quickly removed, washed free of blood, and placed in the buffer solution. Slices of approximately equal size and thickness were made with a razor blade held against the lower side of a glass microscope slide. The sections then were placed in the main chamber of the manometer flasks. At the conclusion of the experiment the slices were removed and dried for twenty-four to forty-eight hours at 75° C. and oxygen consumption was calculated per milligram of dry weight of tissue.

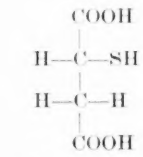
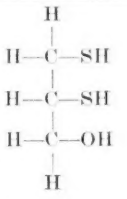
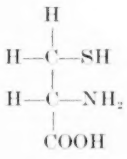
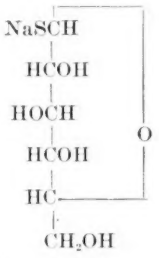
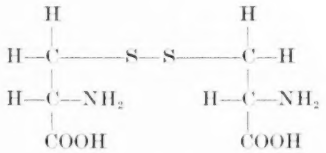
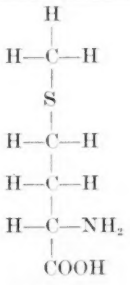
The solutions to be tested were placed in the side arms at 12.5 times the final concentration and tipped into the main chamber containing the tissues after a control period of forty-five minutes. In all cases the final gold concentration was M/500 with respect to gold, since this had been shown to be the lowest concentration which would produce maximal inhibition of oxygen consumption.[6] The thiol compounds were added in concentrations to provide either one thiol or three thiol groups for one atom of gold. This was done since it is possible for gold to react either as a univalent or trivalent ion. The pH of each added solution was adjusted so that the final pH of the solution in the flasks after addition was 7.4.

Each set of experiments included a control tissue slice, one to which gold was added, one with an added thiol compound, and one with both a gold and a thiol compound added. Readings of the manometers were made at fifteen- and thirty-minute intervals, and were continued from one hour and forty-five minutes to two hours and forty-five minutes after addition of solutions being tested.

OBSERVATIONS

The compounds studied are listed in Table I. The inorganic gold salts, gold chloride and gold sodium thiosulfate, were previously shown to have a marked inhibitory effect on the oxygen consumption of rat tissues.[6] Cystine and methionine were included since it was felt that they might be broken down to give compounds with a thiol group.

TABLE I. COMPOUNDS STUDIED

GOLD COMPOUNDS	
$AuCl_3 \cdot HCL \cdot 3H_2O$ Gold chloride (50 per cent gold)	$Na_3Au(S_2O_3)_2$ Gold sodium thiosulfate (37.4 per cent gold)
THIOL COMPOUNDS	
 Thiomalic acid	 2,3-Dimercaptopropanol (BAL)*
 Cysteine	 Sodium thioglucose†
POTENTIAL THIOL COMPOUNDS	
 l-Cystine	 Methionine

*The BAL was obtained from Edgewood Arsenal, Md., through courtesy of the United States Army.

†Sodium thioglucose was furnished by the Schering Corporation, Bloomfield, N. J.

Only the results with kidney slices are included in this report. Essentially the same results were obtained with liver slices; however, since the oxygen consumption of normal liver tissue is smaller than that of kidney tissue the absolute differences noted with the compounds studied are much smaller than those found in the kidney.

Oxygen consumption of rat kidney slices, as influenced in vitro by the gold compounds alone, by thiol and di-thiol compounds alone, and by a combination of each, is shown in Fig. 1 for gold sodium thiosulfate and Fig. 2 for gold

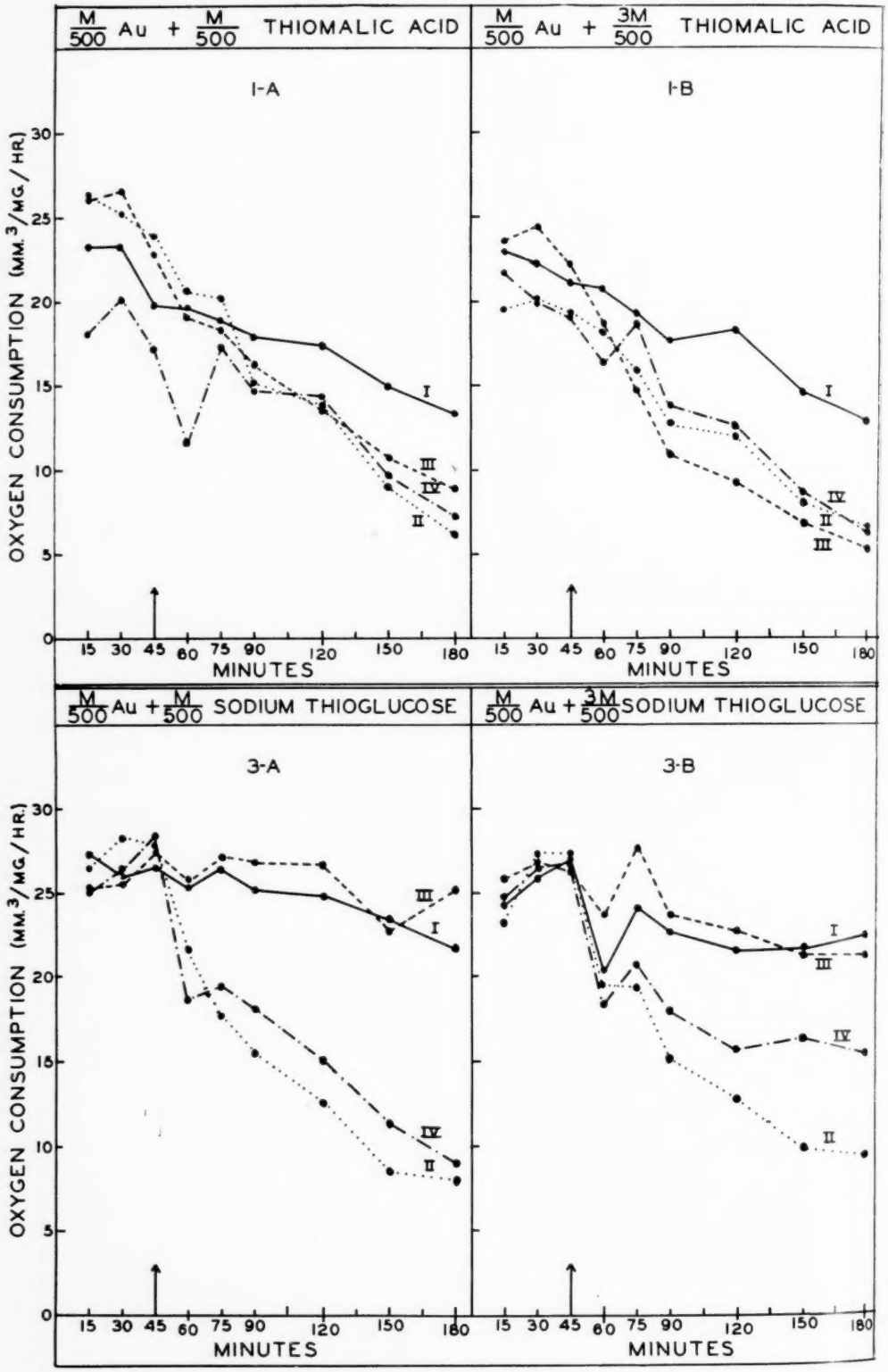


Fig. 1.—Oxygen consumption of rat kidney slices, as influenced in vitro by gold sodium thiosulfate alone, by thiol and di-thiol compounds alone, and by a combination of each. I ——, No compounds added. II . . . . . , Gold sodium thiosulfate added. III – – –, Thiol compounds added. IV –.–., Gold and thiol compounds added. The vertical arrow indicates time of addition of compound tested.

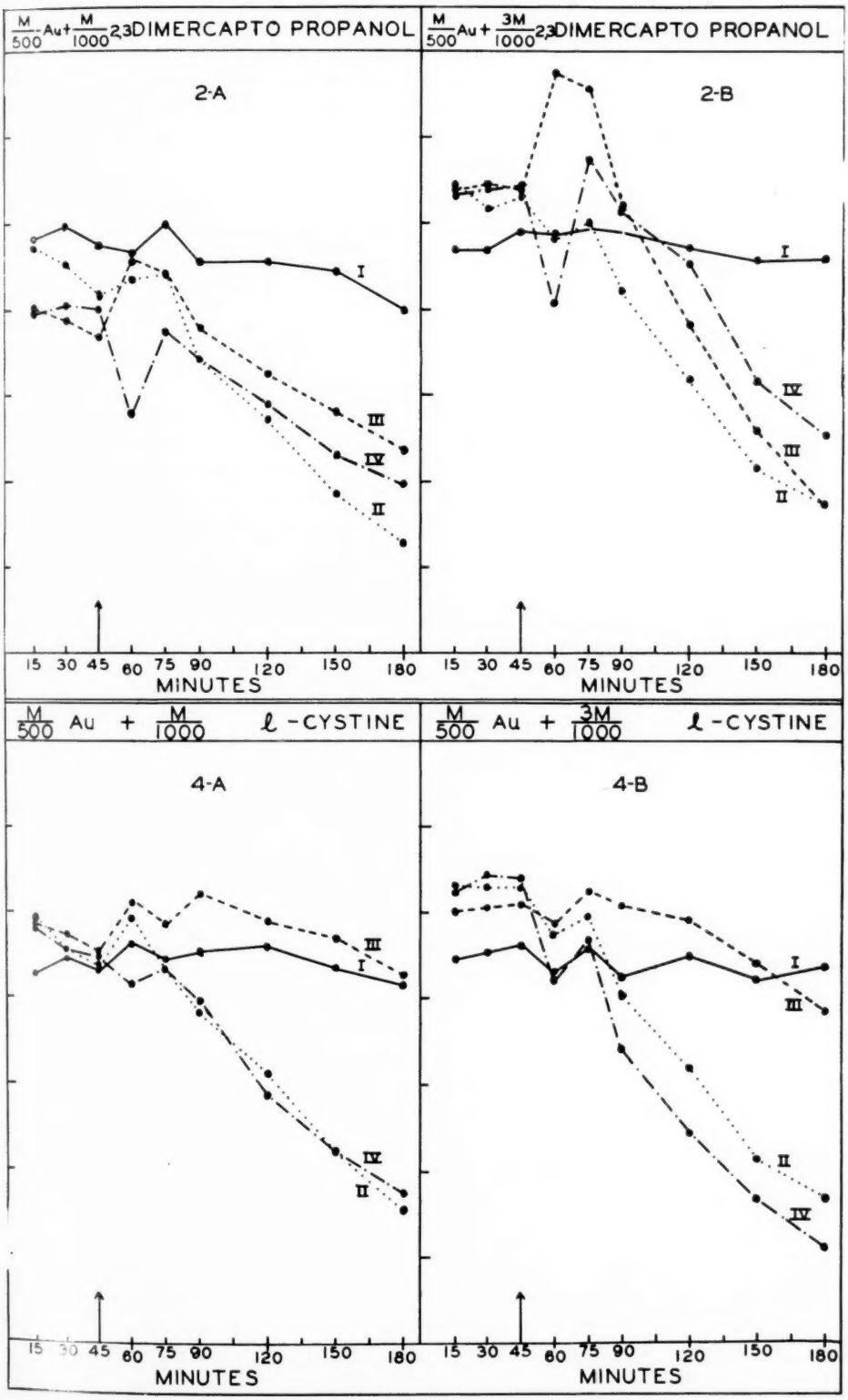


Fig. 1.—Continued. *See legend on opposite page.*

chloride. Oxygen consumption is plotted on the vertical axis against time in minutes on the horizontal axis. The arrow at forty-five minutes indicates the time of additions to the flask. Each curve represents the average of six to eight determinations. The control curve is shown by the solid line, the gold by a dotted line, thiol compound by a broken line, and both compounds by a combination dot-dash line. On the left, in each pair of experiments, the chart shows the results obtained when one thiol group was provided per atom of gold; on the right, the curves represent results with three thiol groups per atom of gold.

Gold Sodium Thiosulfate (Fig. 1).—The first pair of curves, *1-A* and *1-B*, shows the effect of thiomalic acid and gold sodium thiosulfate on oxygen consumption of rat kidney slices. The control curves (I) in both sets of experiments show a gradual decline in respiration over the three-hour period. In the M/500 concentration *(1-A)* thiomalic acid (III) produces an inhibition of respiration somewhat less than that produced by M/500 gold (II). The addition of both compounds simultaneously to normal tissue results in a respiration (IV) slightly higher than when gold alone is added (II) but lower than with thiomalic acid alone (III). In the 3M/500 concentration *(1-B)* thiomalic acid itself (III) produces an inhibition of respiration greater than that caused by gold alone (II). However, the simultaneous addition of both compounds results in a curve (IV) which follows very closely that of gold alone (II), indicating that in this instance the inhibition of respiration is not the additive effect of the inhibition produced by each compound alone.

The effect of BAL (2,3-dimercaptopropanol) is shown in *2-A* and *2-B*. In both sets of experiments the control curves (I) show little decrease in respiration over the whole period. In the M/1,000 concentration *(2-A)*, where one thiol group is provided per atom of gold, the BAL alone (III) produces less inhibition of respiration than does gold alone (II). The curve for the addition of both compounds (IV) shows less inhibition than when gold alone is added (II), but greater inhibition than that caused by the thiol compound alone (III). In the 3M/1,000 *(2-B)* BAL itself (III) produces an inhibition comparable to that of gold alone (II); however, the rate at which respiration decreases is slower with BAL than with gold. Tissue respiration after the addition of both gold and BAL (IV) is inhibited as compared with the control (I), but continues at a somewhat higher level than with either compound alone. Here again, the inhibitory effect of both compounds is not the sum of the effect of each alone, and it is possible that there is a greater prevention of respiratory inhibition caused by gold which is masked by the individual inhibitory action of either compound.

The influence of sodium thioglucose and gold sodium thiosulfate is shown by the curves in *3-A* and *3-B*. In concentrations of M/500, equimolar with respect to gold, the thiol compound (III) has no effect when compared with control tissue respiration (I). Its addition together with gold results in a respiration curve (IV) which shows definite inhibition though it is less than that produced by the addition of the gold salt (II). In the 3M/500 concentration, sodium thioglucose produces no inhibition of oxygen consumption (III)

when compared with the control respiration rate (I). However, the addition of both thiol and gold compounds results in a respiration curve (IV) which is significantly higher than that obtained when only gold is added (II).

The last pair of curves, *4-A* and *4-B*, Fig. 1, presents results obtained with l-cystine, a potential di-thiol compound. In both sets of experiments, cystine itself produces respiration curves (III) which are similar to the control curves (I). In the M/1,000 concentration, equimolar with respect to gold, cystine added simultaneously with gold (IV) produces an inhibition of respiration comparable with that caused by the gold salt alone (II). In the 3M/1,000 concentration, the addition of cystine with gold (IV) results in an even lower respiration rate than that produced by gold alone (II). Despite the fact that the pH of all solutions added was adjusted so that the pH of the reaction media was 7.4 after additions to the main chamber, we found that the gold solution plus the 3M/1,000 cystine resulted in a pH of over 8.0 at the conclusion of the experiment. This fact may account in these experiments for the observed respiratory inhibition which is greater than that caused by the gold salt alone.

Methionine, not shown in the figure, gave essentially the same results as l-cystine.

Gold Chloride (Fig. 2).—The in vitro effect of gold chloride and the various thiol compounds studied on the respiration of rat kidney slices is shown in Fig. 2. The first set of experiments, *1-A* and *1-B*, shows the influence of thiomalic acid. Alone, in M/500 concentration, this thiol compound has a depressant effect on respiration as shown by curve III; however, when it is added with the gold salt, the respiration rate (IV) is somewhat higher than that produced by the gold alone (II), though lower than with thiomalic acid alone (III). In the 3M/500 concentration *(1-B)*, thiomalic acid and the gold salt added simultaneously produce a respiration curve (IV) which shows definitely less inhibition of oxygen consumption than when either compound is added alone. There is, however, definite inhibition when this respiration rate is compared with the control (I).

Essentially the same results were obtained with BAL and gold chloride, *2-A* and *2-B*, Fig. 2, as with thiomalic acid and gold chloride. In the 3M/1,000 concentration *(2-B)*, BAL shows a greater inhibition of respiration alone (III) than in the M/1,000 concentration (III, *2-A*), but its ability to prevent inhibition is much more marked (IV, *2-B*) at this concentration than at M/1,000 (III, *2-A*).

Both sodium thioglucose and l-cystine behave similarly, *3-A* and *3-B*, and *4-A* and *4-B*, respectively. Neither compound produces inhibition of itself (III). Each, when added with the gold chloride in concentrations to provide one thiol group per atom of gold, shows some prevention of inhibition (IV, *3-A* and *4-A*). In the larger concentration, three thiol groups per atom of gold, each compound shows a greater prevention of inhibition (IV) than when only one thiol group is provided per gold atom.

Methionine and cysteine were also studied. Neither produced inhibition of oxygen consumption by itself, nor was there any appreciable prevention of the inhibition produced by gold chloride.

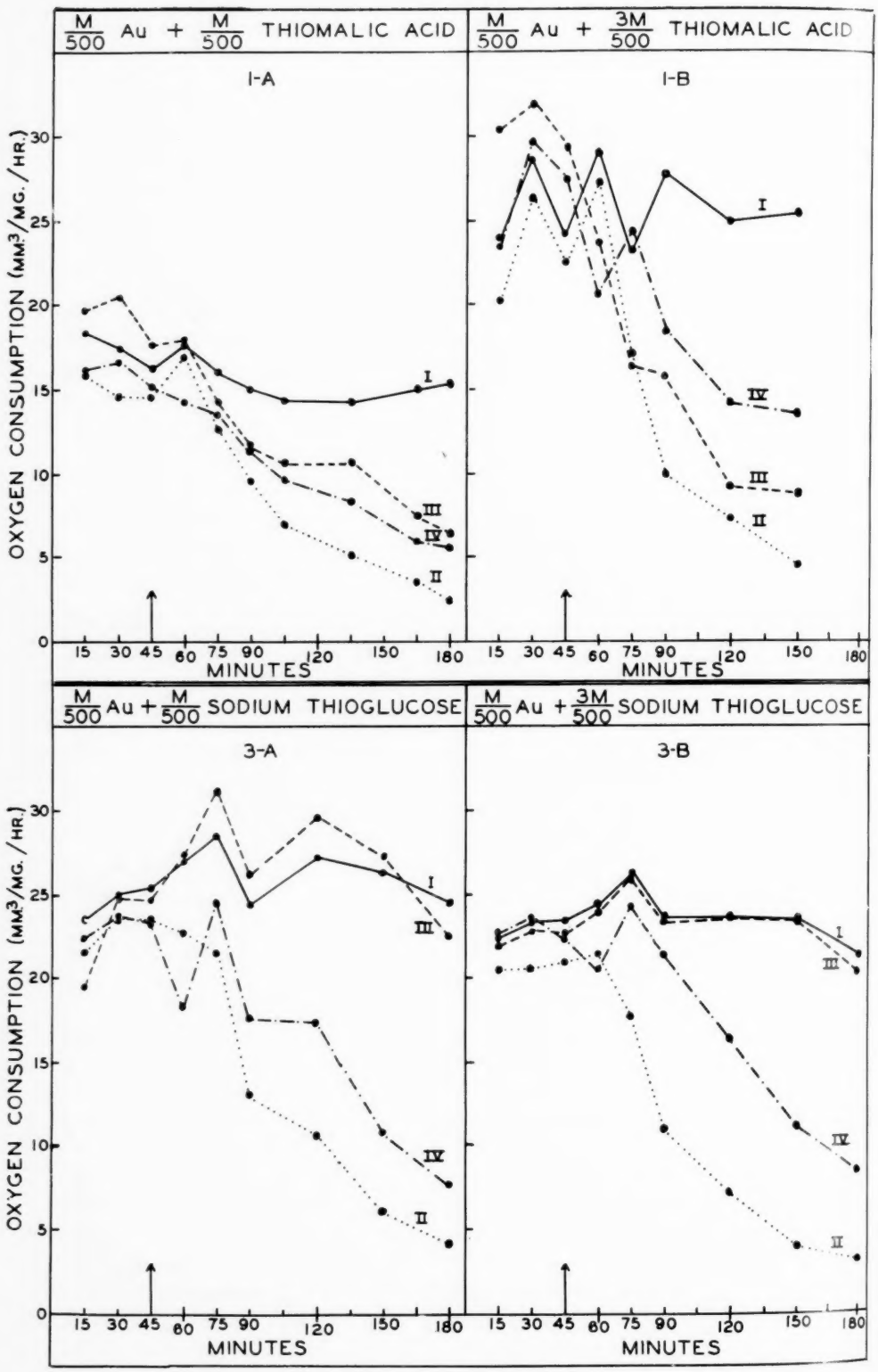


Fig. 2.—Oxygen consumption of rat kidney slices, as influenced in vitro by gold chloride alone, by thiol and di-thiol compounds alone, and by a combination of each. I ——, No compounds added. II . . . ., Gold chloride added. III – – –, Thiol compounds added. IV – . – ., Gold and thiol compounds added. The vertical arrow inicates time of addition of compound tested.

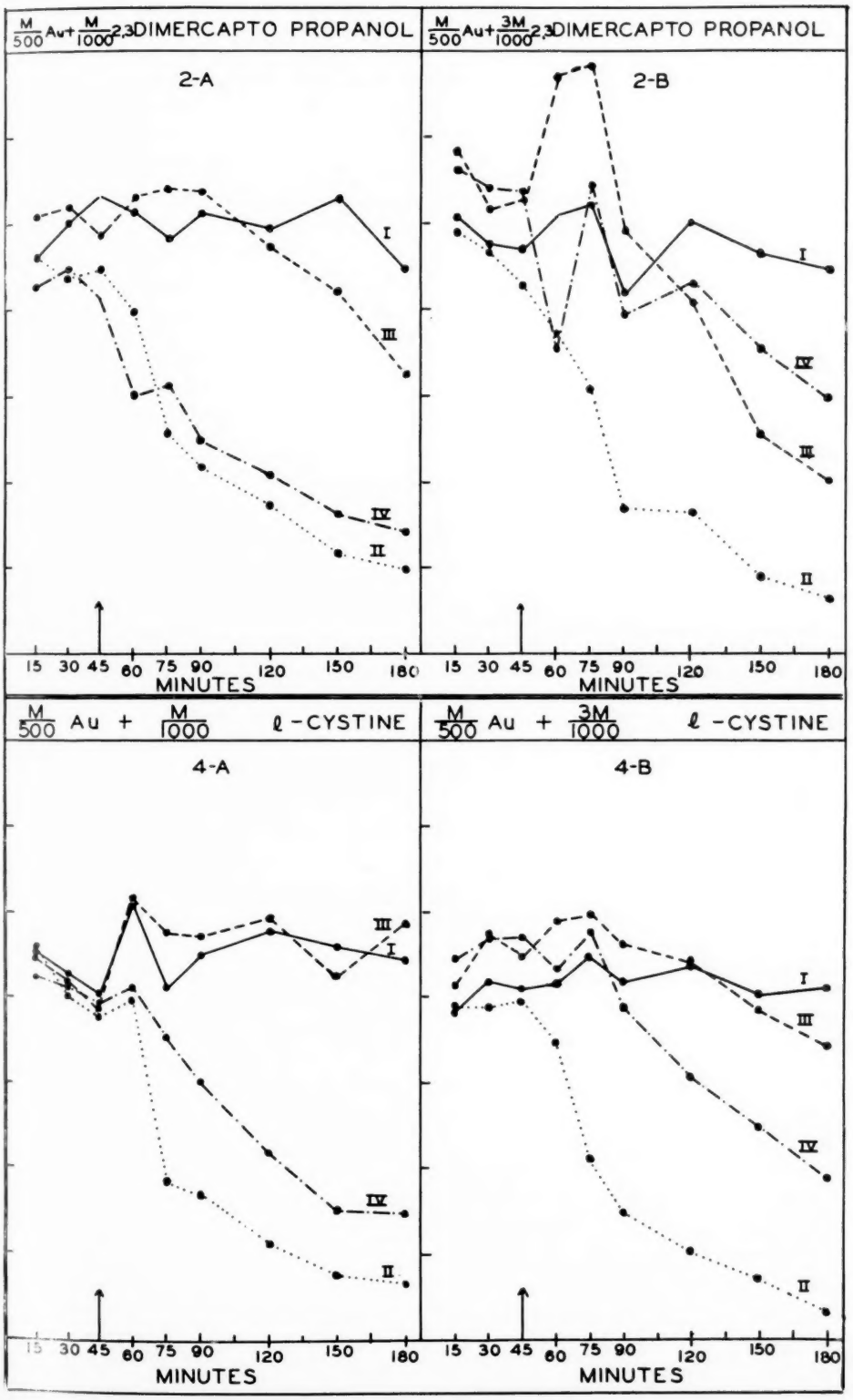


Fig. 2.—Continued. *See legend on opposite page.*

DISCUSSION

With the exception of sodium thioglucose, none of the compounds containing a thiol or a potential thiol group definitely affected the inhibition in oxygen consumption caused by gold sodium thiosulfate. Yet all compounds except methionine and cysteine were effective against gold chloride. This could be most clearly demonstrated when three thiol groups were furnished for each atom of gold. However, both BAL and thiomalic acid produced an inhibition of oxygen consumption, when added alone, which was not evident with the other thiol compounds studied. The simultaneous addition of either of these with the gold sodium thiosulfate did not result in an additive effect with respect to respiratory inhibition. When either of these two was added with gold chloride, there was definitely less inhibition than was produced by either the thiol compound or the gold salt alone.

It appears that under conditions of these experiments, there is a chemical reaction in vitro between gold chloride and thiol groups to produce a compound which does not inhibit tissue respiration. The gold compounds theoretically formed, for example, gold thiomalate and gold thioglucose, were shown not to inhibit oxygen consumption in previous studies.[6] Postulated compounds formed by the reaction of gold chloride and BAL or l-cystine would be expected to behave similarly. We have no explanation for the failure of cysteine and methionine to counteract inhibition.

The results with gold sodium thiosulfate are not as clear cut. Because of the complicated chemical nature of this salt, it is difficult to postulate what compounds might be formed by the thiol derivatives.

The ability of thiol groups to remove gold deposited in tissues has not been investigated by this study. The work here presented has shown the ability of thiol compounds to lessen the in vitro inhibition of oxygen consumption when the compounds are used simultaneously with certain gold salts.

The implications of these studies with respect to the therapeutic uses of gold are not clear. Gold chloride is extremely toxic and has not been used in treating human beings. Yet it is the only salt which, under the conditions of this study, appears to be detoxified by various thiol groups. The inhibitory action of gold sodium thiosulfate is prevented only by sodium thioglucose, and not by the other thiol groups studied; some detoxifying action by BAL and sodium thiomalate may be masked by the fact that these compounds per se inhibit oxygen consumption.

The gold salts commonly used in the treatment of rheumatoid arthritis, gold sodium thiomalate and gold thioglucose, do not interfere in vitro with tissue respiration. Since in these two preparations the gold is bound to the organic moiety by a sulfur linkage, it is possible that the empiric development of therapeutic gold preparations has already taken advantage of the thiol detoxifying mechanism. This interpretation cannot be proved by in vitro studies, since it is possible that gold salts are metabolized by a common pathway, regardless of the form in which they are introduced into the body. Also, the failure of sodium succinimido-aurate, which contains no sulfur, to interfere with tissue respiration[6] suggests that factors other than sulfur linkage may be important in determining toxicity.

SUMMARY

1. The in vitro inhibition of oxygen consumption of rat kidney slices observed with gold sodium thiosulfate is not lessened by the potential thiol compounds cystine and methionine, nor by BAL or thiomalic acid which contain thiol groups; however, sodium thioglucose does lessen the inhibition of oxygen consumption. The effects of BAL and thiomalic acid may be masked by the fact that these compounds, in themselves, produce inhibition of respiration.

2. The in vitro inhibition of oxygen consumption of rat kidney slices caused by gold chloride is appreciably reduced by thiomalic acid, BAL, l-cystine, and sodium thioglucose, but not by methionine or cysteine.

3. When three thiol groups are furnished for each atom of gold, the reduction of inhibition is more clearly shown than when only one thiol group per atom of gold is present.

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THE EFFECT OF PARA-AMINOBENZOIC ACID ON THE METABOLISM AND EXCRETION OF SALICYLATE

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THE elevation of plasma salicylate levels, obtained by the ingestion of a fixed dose of salicylate, after the oral administration of para-aminobenzoic acid was reported in 1946 by Dry, Butt, and Scheifley.[1] In view of the wide use of salicylates in medicine, further study in an effort to determine the mechanism by which oral administration of para-aminobenzoic acid increases the plasma salicylate level seemed justified.

METHOD AND EXPERIMENTAL PROCEDURE

Para-aminobenzoic acid was determined in the blood and urine by the Bratton-Marshall[2] method and plasma salicylate was determined by the method of Brodie, Udenfriend, and Coburn.[3] The salicyl fractions in the urine were determined by the method described by Smith and co-workers.[4] Quick's[5] method was used to determine the hippuric acid in the urine. The unbound or filtrable portion of the plasma salicylate was determined by the method described by Lester, Lolli, and Greenberg.[6]

All blood was drawn in heparin-moistened syringes and centrifuged, and the plasma was separated from the cells as soon as possible. Five healthy men between the ages of 26 and 32 years and three normal, trained dogs were used in this study.

The effects of oral administration of para-aminobenzoic acid on the plasma salicylate levels and on the urinary excretion of salicylate, after oral administration of a single dose of sodium salicylate, were studied in both man and the dog. In the studies on man, the subject was given 3 Gm. of sodium salicylate at 8:00 A.M. and blood samples were drawn at intervals over the next fifty-six hours. After the ingestion of salicylate, all urine passed during the remainder of the study was collected and immediately refrigerated at 0° C. until the salicyl fractions were determined. When para-aminobenzoic acid was used, its administration was started eighteen hours before the ingestion of salicylate and 3.0 Gm. of para-aminobenzoic acid were given every three hours for the duration of the study.

In similar studies on dogs the procedure was much the same. One gram of sodium salicylate was given orally or 0.1 Gm. of sodium salicylate per kilogram of body weight was given intravenously and the animals were placed in a metabolism cage. Blood samples were drawn at intervals over the next thirty hours. The urine was collected under toluene in four-hour samples and refrigerated at 0° C. When para-aminobenzoic acid was used, its administration was started eighteen hours before the administration of salicylate and 0.5 Gm. of para-aminobenzoic acid was given orally every three hours for the duration of the study.

In clearance studies on human subjects, two sixty-minute specimens of urine were collected and one blood sample was drawn at the middle of each collection period. The clearance was calculated for each period and the two values were averaged. The subject was given 3.0 Gm. of sodium salicylate orally for the clearance test and when para-aminobenzoic acid was used it was given as described previously. The pH of the urine was determined as soon as possible on a Beckman pH meter and the specimen was then refrigerated at 0° C.

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The oral hippuric acid liver function test was carried out in the manner described by Quick and commonly used in clinical medicine. As in the foregoing procedures, when para-aminobenzoic acid was used, its administration was started eighteen hours before the ingestion of sodum benzoate and it was given every three hours. When glycine was used, 20 Gm. were given with breakfast and 20 Gm. with the sodium benzoate.

RESULTS

When three subjects of similar height and weight were given orally a single 3 Gm. dose of sodium salicylate, the plasma salicylate level reached its peak of 18 to 22 mg. per 100 c.c. in two to four hours and then fell rapidly to 1 mg. or less per 100 c.c. in twenty-eight to thirty-two hours. However, when the procedure was repeated and para-aminobenzoic acid was also given, the plasma salicylate level again reached its peak in two to four hours but fell more slowly. In one subject the plasma salicylate level dropped to 1 mg. per 100 c.c. in forty-eight hours, and in the other two subjects it was more than 3 mg. per 100 c.c. at fifty-six hours. Fig. 1 shows results obtained on Subject 4 plotted on arithmetic graph paper and Fig. 2 shows the same results plotted on semilogarithmic paper. Oral administration of para-aminobenzoic acid appeared to cause the plasma salicylate levels to fall more slowly and to alter the form of the curve.

Table I shows the salicyl fractions found in the urine of Subject 4 in the foregoing study. In man, after oral administration of the single dose of salicylate, there were the expected large quantities of salicyluric acid present in the urine. However, when para-aminobenzoic acid was given with the salicylate, only very small quantities of salicyluric acid were found in the urine in spite of collection of the urine for a period almost twice as long. The quantities of free salicylate and salicyl glucuronate were comparable when the duration of the collection period was considered. There was no retention of salicyluric acid in the blood when it failed to appear in the urine, as the plasma salicylate was practically all in the form of free salicylate and only the usual traces of salicyluric acid were present.

TABLE I. THE SALICYL FRACTIONS RECOVERED FROM THE URINE OF SUBJECT 4 AND DOG 261 IN THE SAME STUDIES ILLUSTRATED BY FIGS. 1 THROUGH 6

	COLLECTING PERIOD (HR.)	SODIUM SALICYLATE	SALICYL FRACTIONS IN THE URINE (MG.)			
			SALICYLURIC ACID	FREE SALICYLATE	SALICYL GLUCURONATES	TOTAL SALICYLATE
Subject 4	34	Without PAB	1,364.36	319.43	298.78	1,982.57
	60	With PAB	71.50	819.39	610.61	1,501.50
Dog 261	48	Without PAB	4.54	185.46	203.95	393.95
	48	With PAB	0	198.0	264.0	462.0

Four similar studies were done on two dogs; the salicylate was given orally twice and intravenously twice. The results on one dog are shown in Figs. 3, 4, 5, and 6. Salicylate excretion was much slower in the dog than in man and when para-aminobenzoic acid was given, the rate of excretion of salicylate was not altered appreciably.

In the dog the administration of para-aminobenzoic acid did not change the salicyl fractions appearing in the urine. Only traces of salicyluric acid were

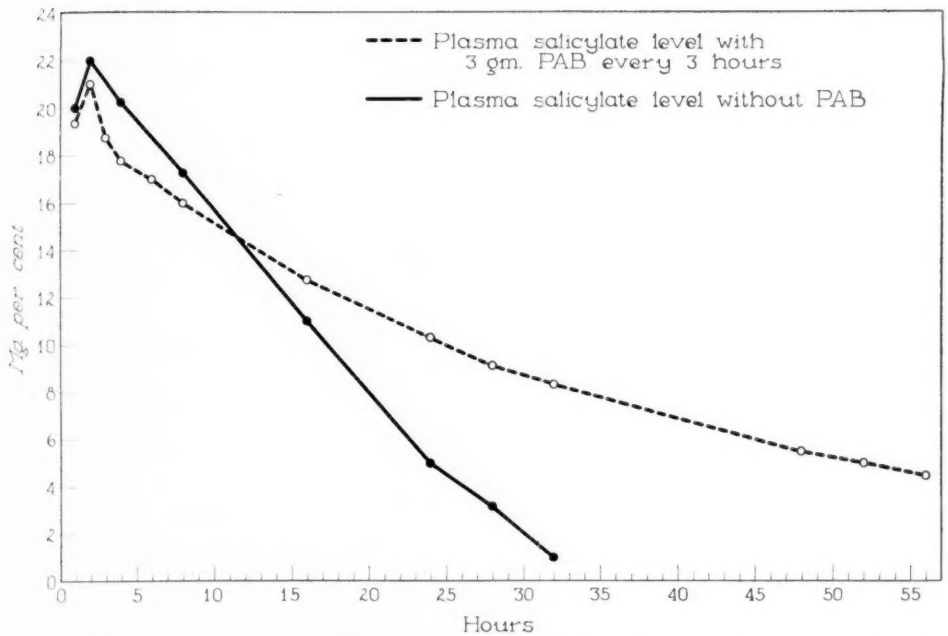

Fig. 1.—Salicylate concentration in the plasma of Subject 4 after administration of a single dose of 3 Gm. of sodium salicylate. Three grams of para-aminobenzoic acid were given every three hours throughout the experiment.

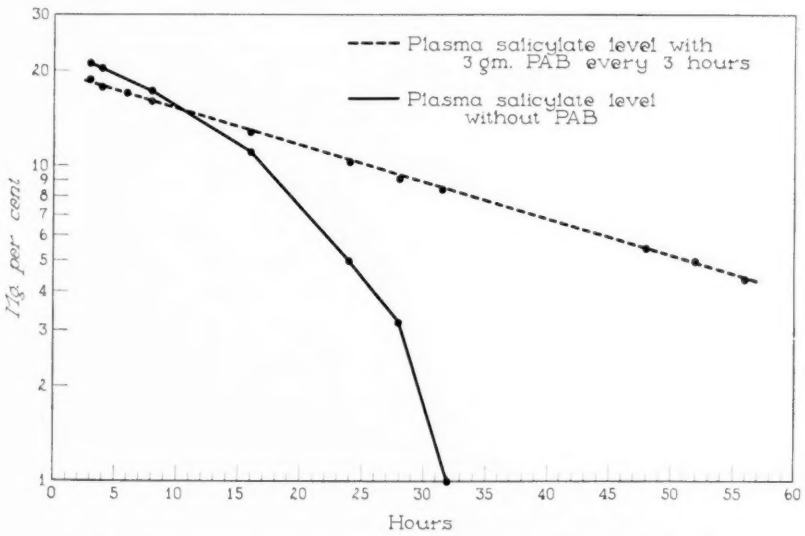

Fig. 2.—The same values as in Fig. 1 plotted on semilogarithmic paper.

present in the urine when salicylate alone was given. Table I shows the salicyl fractions found in the urine of Dog 261 in the foregoing studies.

Table II shows the renal clearance figures obtained on four human subjects. The clearance values of salicylate without para-aminobenzoic acid ranged from 50.46 to 99.36 c.c. of plasma cleared per minute, with an average of 70.29 cubic

centimeters. The clearance values of salicylate when para-aminobenzoic acid was given ranged from 22.76 to 32.41 c.c. of plasma cleared per minute, with an average of 27.53 cubic centimeters. The renal clearance of salicylate apparently

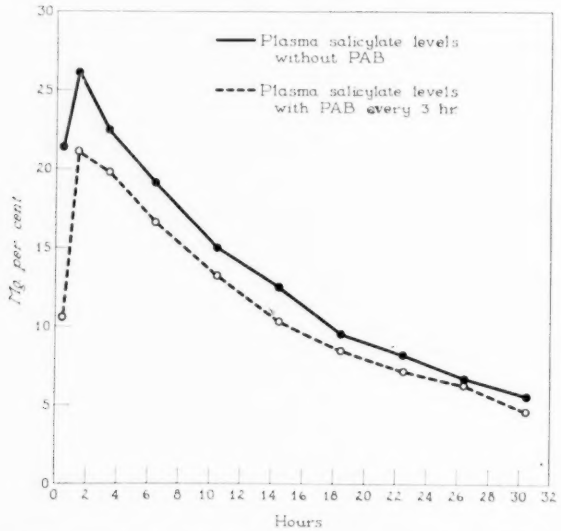

Fig. 3.—Salicylate concentration in the plasma of Dog 261 after the oral administration of 1 Gm. of sodium salicylate. One-half gram of para-aminobenzoic acid was given every three hours throughout the study.

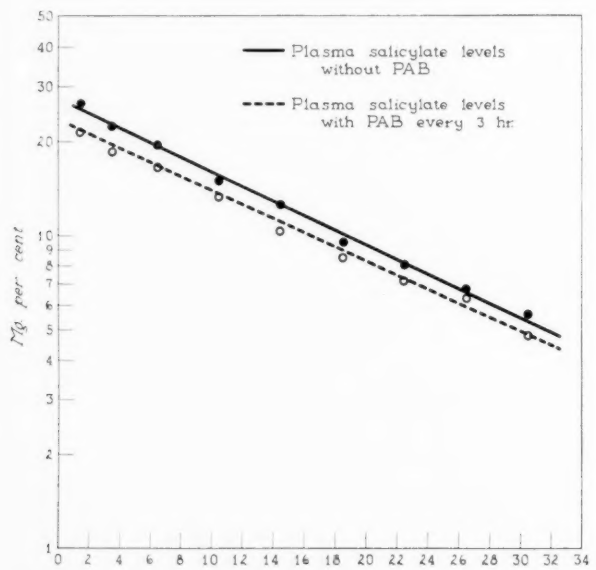

Fig. 4.—The values in Fig. 3 plotted on semilogarithmic paper.

was reduced approximately 60 per cent when para-aminobenzoic acid was given. However, in calculating these clearance values the urine was hydrolyzed and the figure for the total salicylate present was used regardless of what salicyl

fractions were present. Since this clearance value is actually the resultant of all the clearance figures of the individual salicyl fractions appearing in the urine, not much information can be obtained from it.

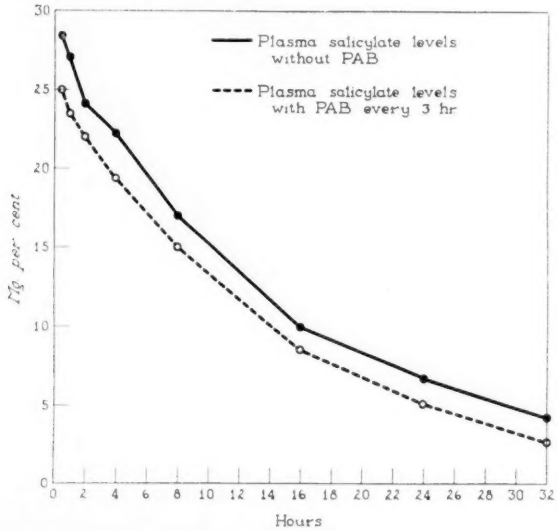

Fig. 5.—Salicylate concentration in the plasma of Dog 261 after the intravenous administration of 0.1 Gm. of sodium salicylate per kilogram of body weight. One-half gram of para-aminobenzoic acid was given every three hours throughout the study.

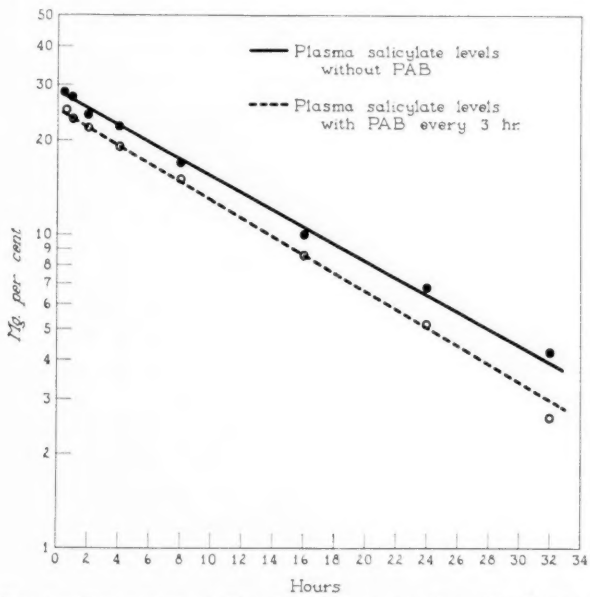

Fig. 6.—The values of Fig. 5 plotted on semilogarithmic paper.

Table III shows the results of another group of clearances in which the salicyl fractions in the urine were determined. When para-aminobenzoic acid was given with the salicylate, again only traces or very small quantities of

TABLE II. DECREASE OF THE RENAL CLEARANCE OF TOTAL SALICYLATE AFTER THE INGESTION OF PARA-AMINOBENZOIC ACID IN MAN; CLEARANCES ARE GIVEN AS CUBIC CENTIMETERS OF PLASMA CLEARED PER MINUTE

SUBJECT	VOLUME OF URINE (C.C. PER MIN.)	TOTAL SALICYLATE CLEARANCE (C.C. PER MIN.)		PLASMA PAB LEVELS (MG. PER 100 C.C.)
		NO PAB	WITH PAB	
1	3.18	77.07		
	1.12	68.70		
	2.70		31.94	12.55
	1.80		32.41	13.23
2	4.65	99.36		
	1.24	73.57		
	2.31		25.41	9.15
	1.26		26.52	8.25
3	1.86	50.46		
	1.56	51.97		
	3.60		22.76	11.14
	1.40		23.13	8.55
4	3.40	77.42		
	1.40	63.75		
	2.75		28.40	10.2
	1.50		29.65	8.6
Average		70.29	27.53	

salicyluric acid appeared in the urine. Since salicyluric acid has a higher renal clearance value than free salicylate and normally constitutes approximately 50 per cent of the total salicyl fractions excreted in the urine, the deficiency of this salicyl fraction caused a marked drop in the renal clearance of the total salicylate. At the same time clearance of the free salicylate fraction remained essentially unchanged unless the pH of the urine was also decreased. However, when enough sodium bicarbonate was given with the salicylate and para-aminobenzoic acid to produce a strongly alkaline urine, the clearance of the free salicylate fraction increased enough to mask completely the effect of the deficiency of

TABLE III. THE EFFECTS OF THE INGESTION OF PARA-AMINOBENZOIC ACID, GLYCINE, AND SODIUM BICARBONATE ON THE EXCRETION AND CLEARANCE OF THE SALICYL FRACTIONS IN MAN

SUBJECT		VOLUME OF URINE (C.C. PER MIN.)	PH OF URINE	SALICYL FRACTIONS IN THE URINE (MG. IN 1 HR.)				RENAL CLEARANCE (C.C. OF PLASMA PER MIN.)	
				SALICYLURIC ACID	FREE SALICYLATE	SALICYL GLUCURONATES	TOTAL SALICYLATE	FREE SALICYLATE	TOTAL SALICYLATE
1	No PAB	1.8	6.8	72.46	30.0	32.61	135.07	17.68	79.93
	With PAB	2.27	6.4	8.31	25.85	34.28	68.44	12.11	32.00
	With PAB plus glycine	2.73	7.5	5.12	117.19	30.38	152.69	61.03	79.50
2	No PAB	1.88	6.9	60.00	32.42	38.64	131.06	19.64	79.42
	With PAB	1.60	6.85	16.94	41.66	40.85	99.45	19.44	46.4
	With PAB plus sodium bicarbonate	2.70	8.0	Trace	197.50	32.5	230.0	106.93	124.57
3	With PAB plus sodium bicarbonate	1.20	7.85	12.4	159.36	33.5	205.26	92.96	119.75
	With sodium bicarbonate	1.75	7.75	68.45	162.25	38.4	269.10	98.33	163.1
4	With PAB plus glycine	2.07	7.6	5.0	114.80	50.65	170.45	62.62	92.99

salicyluric acid on the total salicylate clearance. In an attempt to see if the quantity of available glycine was the limiting factor, glycine was given with the para-aminobenzoic acid and salicylate, but still only small quantities of salicyluric acid were found in the urine. The urine was consistently alkaline when glycine was ingested and the excretion of total salicylate was high because of the increased renal clearance of the free salicylate fraction.

TABLE IV. DECREASE OF THE FORMATION OF HIPPURIC ACID IN MAN AFTER THE INGESTION OF PARA-AMINOBENZOIC ACID AS INDICATED BY THE ORAL HIPPURIC ACID LIVER FUNCTION TEST

SUBJECT	DAY (JUNE, 1947)	GRAMS OF HIPPURIC ACID EXCRETED IN THE URINE IN FOUR HOURS AFTER ADMINISTRATION OF		
		6 GM. SODIUM BENZOATE WITH PAB	6 GM. SODIUM BENZOATE WITHOUT PAB	6 GM. SODIUM BENZOATE WITH PAB PLUS GLYCINE
1	25	0.398		
	28		4.980	
	30			0.650
2	25	0.542		
	28		4.743	
	30			0.400
3	30			0.485

In order to see if para-aminobenzoic acid was capable of interfering with the conjugation of glycine with benzoic acid or its derivatives, other than salicylic acid, the hippuric acid liver function test was used. Table IV shows that there was a great decrease of the formation of hippuric acid when para-aminobenzoic acid was given, and the exogenous glycine did not eliminate this effect.

COMMENT

The results of this study substantiate the finding of Dry, Butt, and Scheifley that the oral administration of para-aminobenzoic acid caused an elevation of the plasma salicylate level in man. This elevation did not occur in the dog. The mechanism responsible for this elevation of plasma salicylate level in man appeared to be an alteration of the detoxication of salicylate.

According to the reports of Bertagnini,[7] Tollens,[8] Quick,[9] Salt,[10] Kapp and Coburn,[11] and Smith and co-workers,[4] man normally conjugates salicylic acid with both glycine and glucuronic acid, and the corresponding products, salicyluric acid and salicyl glucuronates, appear in the urine. Kapp and Coburn reported that approximately 80 per cent of the ingested salicylate was excreted in the urine as compounds containing the salicyl radicle and roughly 50 per cent of this was salicyluric acid. This conjugation probably occurs in the liver. Quick[12, 13] has pointed out that the liver of the dog does not conjugate glycine with benzoic acid and its derivatives to the same extent as the liver of man and many animals, but does use glucuronic acid extensively in the conjugation of benzoic acid and orthohydroxybenzoic acid.

Working with rabbits, Ellinger and Hensel[14, 15] in 1914 first reported the excretion of acetyl-para-aminobenzoic acid after the feeding of para-aminobenzoic acid. After this report, para-aminobenzoic acid was widely used in the study of some of the mechanisms of detoxication in man as well as animals, and

the literature on the metabolism of the drug is extensive.[16-27] These reports indicate that, in man, para-aminobenzoic acid is conjugated with glycine and glucuronic acid and that the corresponding products appear in the urine in addition to acetyl-para-aminobenzoic acid.

The findings in this study are in agreement with the foregoing observations in that only traces of salicyluric acid were found in the urine of the dogs after the administration of salicylate, while in man large quantities of salicyluric acid were excreted in the urine after the ingestion of salicylate. When para-aminobenzoic acid was given to man with sodium salicylate, only traces or very small quantities of salicyluric acid appeared in the urine. Since there was no retention of salicyluric acid in the blood when it failed to appear in the urine, its formation was apparently interrupted or markedly decreased. Although the principal mechanism by which para-aminobenzoic acid increased the plasma salicylate level and decreased total salicylate excretion appeared to be the interruption of salicyluric acid formation, para-aminobenzoic acid also tended to lower the pH of the urine and thus further decreased the total salicylate excretion by decreasing the renal clearance of the free salicylate fraction. When the urine was made strongly alkaline by the administration of massive doses of sodium bicarbonate, the clearance of the free salicylate fraction increased, as observed by Smith and co-workers,[4] and the effect of para-aminobenzoic acid was masked.

Because of the fact that no relatively simple methods were available for determining the concentrations of the conjugated products of para-aminobenzoic acid in the urine or blood, no attempts were made to study the possible reciprocal effects of salicylic acid on the detoxication or excretion of para-aminobenzoic acid.

SUMMARY

The oral administration of para-aminobenzoic acid appeared to have the following effects on the metabolism and excretion of salicylate in man. First, it altered the detoxication of salicylate by interrupting or greatly depressing the conjugation of glycine with salicylate so that only very small quantities of salicyluric acid appeared in the urine after ingestion of salicylate. Second, it tended to lower the pH of the urine and thus decreased the renal clearance of the free salicylate fraction. Third, it caused a decrease of the urinary excretion of total salicylate and a rise of the plasma salicylate level due to the foregoing effects.

In dogs the administration of para-aminobenzoic acid did not alter the excretion of salicylate and did not increase the plasma salicylate levels.

The administration of para-aminobenzoic acid appeared to decrease greatly the formation of hippuric acid as measured by the oral hippuric acid liver function test in man.

These effects were temporary and completely reversible.

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USE OF PENICILLIN AND STREPTOMYCIN IN THE ISOLATION OF POLIOMYELITIS VIRUS FROM FECAL SPECIMENS

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IN ORDER to help confirm the diagnosis of poliomyelitis that had been made in several epidemics during the past two years, it was necessary to recover the virus from fecal specimens. Melnick[1] has recommended methods of preparation by high-speed centrifugation, but because the instruments were not available, the etherization method of Paul and Trask[2] was used. Isolations of the virus were made in two epidemics but because so often the monkeys succumbed to peritonitis after several intra-abdominal inoculations, another method was sought to reduce the bacterial content without loss of virus activity.

It had been shown by various workers that the antibiotics penicillin and streptomycin have little effect on the virulence of certain viruses, while on the other hand they greatly reduce the associated bacteria. Parker and Diefendorf[3] reported that the viruses of vaccinia, St. Louis encephalitis, and of equine encephalomyelitis were able to grow in the chicken embryo after addition of penicillin. Florman, Weiss, and Council[4] had used streptomycin without any effect against influenza A virus; Hirst[5] likewise found penicillin to be of use in the isolation of the latter virus, while Hodges[6] obtained marked reduction of bacteria after the simultaneous inoculation of penicillin, streptomycin, and fecal specimen into eggs. Bradley and co-workers[7] employed penicillin to inhibit bacterial growth in the isolation of Newcastle disease virus from contaminated chicken tissues, while Beaudette, Bivins, and Miller[8] have recently advocated the use of a mixture of penicillin and streptomycin for the recovery of the latter virus from chickens. Because the organisms were inhibited and yet none of the viruses used were particularly affected by these antibiotics, the following studies were undertaken with the virus of poliomyelitis. Before testing the antibiotics directly on fecal specimens, it seemed advisable to determine both what effect penicillin and streptomycin might have on different dilutions of poliomyelitis virus and what might be the optimal concentrations for inhibiting bacterial growth.

Experiment 1. To Determine the Effect of Streptomycin and Penicillin on Different Dilutions of the MV Virus of Poliomyelitis.—A 10 per cent aqueous suspension of monkey cord containing the MV† monkey passage virus of poliomyelitis was centrifugated at 6,000 revolutions per minute for thirty minutes in the angle centrifuge placed in the cold room. The supernatant fluid was diluted

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TABLE I. EFFECT OF PENICILLIN AND STREPTOMYCIN ON MV POLIOMYELITIS VIRUS ALONE AND ON A MIXTURE OF VIRUS AND BACTERIA

EXPERIMENT	RHESUS MONKEY	MATERIAL INOCULATED	ROUTE OF INOCULATION*	DILUTION OF VIRUS	RESULTS
1	M-148	MV virus and antibiotics	i.n. and i.abd.	1-10	Paralyzed
	M-149	MV virus and antibiotics	i.n. and i.abd.	1-100	Paralyzed
	M-116	MV virus and antibiotics	i.n. and i.abd.	1-500	Paralyzed
	M-147	MV virus alone	i.c.	1-10	Paralyzed
2	M-121	MV virus, fly suspension, and antibiotics	i.n. and i.abd.	1-10	No symptoms. Withstood challenge dose of virus
	M-122	MV virus, fly suspension, and antibiotics	i.n. and i.abd.	1-100	Paralyzed
	M-123	MV virus, fly suspension, and antibiotics	i.n. and i.abd.	1-1000	Paralyzed
	M-124	MV virus, fly suspension and antibiotics	i.n. and i.abd.	1-10,000	No symptoms. Withstood challenge dose of virus
	M-150	MV virus, fly suspension, and antibiotics	i.c.	1-10	Paralyzed
	M-151	MV virus alone	i.c.	1-10	Paralyzed
3	M-137	MV virus, normal feces, and antibiotics	i.n. and i.p.	1-10	Paralyzed
	M-138	MV virus, normal feces and antibiotics	i.n. and i.p.	1-100	No symptoms
	M-139	MV virus, normal feces, and antibiotics	i.n. and i.p.	1-1000	No symptoms. Withstood challenge dose of virus
	M-136	MV virus, normal feces, and antibiotics	i.c.	1-10	Paralyzed
	M-152	MV virus alone	i.c.	1-10	Paralyzed

*i.n., Intranasal instillation, total of 15 ml.; i.abd., intra-abdominal inoculation, total of 30 ml.; i.c., intracerebral inoculation of 1 milliliter.

1:100 and 1:500, and 15 ml. of each dilution were frozen for future use. The antibiotics were then added to the remainder of the three dilutions (1:10, 1:100, and 1:500) in such a concentration that each contained 1,250 units of penicillin and 25 mg. of streptomycin per milliliter. After remaining one-half hour at room temperature, Rhesus monkeys were inoculated intra-abdominally with 16 ml. of each treated dilution of virus and intranasally with 3 ml. of each that remained untreated. Daily injections were made until a total of 32 ml. of each material was given by the first route and 15 ml. by the second. One milliliter of the 10 per cent suspension of MV virus was given intracerebrally to a fourth monkey as a control. All of the animals became completely paralyzed as shown in Table I, Experiment 1. It was apparent that the antibiotics in these concentrations did not reduce the potency of MV virus diluted at least 1:500. In fact the results were more effective than when this same lot of MV virus was titrated in monkeys without either material. With intracerebral inoculations of 1 ml. of each tenfold dilution from 10^{-1} through 10^{-5}, only the monkey receiving the first

dilution became completely paralyzed. However, when given larger amounts by the combined intranasal and intra-abdominal routes, both the animals receiving the 10^{-1} and the 10^{-3} dilutions showed typical paralysis.

Experiment 2. To Determine the Effect of Penicillin and Streptomycin on a Mixture of MV Poliomyelitis Virus and Bacteria Found in Flies.—To test the effect of the antibiotics on different virus concentrations in the presence of bacteria, tenfold dilutions of the MV strain were made in a suspension of flies that had been taken at the city dump. After centrifugation for thirty minutes at 6,000 revolutions per minute, each dilution was treated with the same concentrations of the antibiotics as used in Experiment 1. Likewise, Rhesus monkeys were inoculated with the same amounts of material given by the same routes as in Experiment 1. At the same time 1 ml. of the treated 1:10 suspension was inoculated into a monkey intracerebrally, while another animal was given a similar dose of the untreated 1:10 virus by the same route.

All but two of the monkeys became paralyzed as shown in Table I, Experiment 2. These two probably had an inapparent infection because both withstood a paralyzing dose inoculated intracerebrally at a later date. Likewise, it was of interest that the monkey (M-150) receiving an intracerebral injection of the treated fly material became typically paralyzed without developing a brain abscess.

Experiment 3. To Determine the Effect of Penicillin and Streptomycin on a Mixture of MV Virus and the Bacteria of a Normal Fecal Suspension.—A 20 per cent suspension of normal feces was made in sterile distilled water. Tenfold dilutions of MV virus were prepared in this material and after a light centrifugation a portion of each dilution was frozen for future use. The remaining dilutions were run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute. Sufficient of each dilution was prepared so that aliquot portions could be frozen and removed when needed. One-half hour before the inoculations a 16 ml. vial of each dilution, 10^{-1}, 10^{-2}, and 10^{-3}, was thawed and the antibiotics quickly were added in an amount so that each dilution of virus contained 1,250 units of penicillin and 25 mg. of streptomycin. Rhesus monkeys were given five daily doses of 3 ml. of the untreated material intranasally and two daily doses of 16 ml. each of the treated dilutions intra-abdominally. One animal was injected with 1 ml. of the 1:10 treated material intracerebrally.

As may be seen in Table I, Experiment 3, the virus diluted with fecal suspension was not as potent as that with the fly material. No paralysis occurred beyond the 1:10 dilution. The same lot of frozen virus was used in both experiments. However, once again typical paralysis was produced by the intracerebral route without any evidence of bacterial contamination.

Experiment 4. Effects of the Antibiotics on Fecal Bacteria.—The following experiments were made to determine the approximate optimal bacteriostatic concentration of the antibiotics which could be used with a 20 per cent fecal suspension.

(1) Penicillin (crystalline sodium G) and streptomycin (calcium chloride complex, Merck) were added to a 20 per cent normal fecal suspension in the concentrations shown in Table II. In one series the antibiotics had little effect upon

TABLE II. BACTERIOSTATIC EFFECT OF PENICILLIN AND STREPTOMYCIN ON A FECAL SUSPENSION

30 MIN. AT ROOM TEMPERATURE		CULTURES			
AMOUNT PER ML.		SUSPENSION UNCENTRIFUGED		SUSPENSION CENTRIFUGED 30 MIN. AT 13,000 R.P.M.	
PENICILLIN G (UNITS)	STREPTOMYCIN (MG.)	BLOOD AGAR	BEEF HEART BROTH	BLOOD AGAR	BEEF HEART BROTH
416.6	6.25			0	Rare Gr.+ rods
595.2	5.95	Gr.– rods* Hem. strep.	Gr.+ rods		
625.0	12.50			0	0
1000.0	20.00	Gr.– rods Hem. strep.	Gr.+ and Gr.– rods		
1000.0	20.00			0	0
1000.0	20.00			0	0
1000.0	20.00			0	0
1190.5	11.90	Gr.– rods Hem. strep.	Gr.+ and Gr.– rods		
1111.0	22.20			0	0
2000.0	40.00	Gr.– rods Hem. strep.			
2105.26	52.60			0	0– Transfer on blood agar Gr.+ rods
2381.0	23.80	Gr.– rods Hem. strep.			
None	None	Much growth	Gr.+ and Gr.– rods	Slight growth	Not inoculated

*Gr.–, Gram negative.
†Gr.+, Gram positive.

the aerobic or anaerobic growth of the bacteria on blood agar and in beef heart broth, respectively, when the suspension was not sufficiently centrifugated, while in the other the results were satisfactory when the material was run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute.

TABLE III. COMPARISON OF THE BACTERIOSTATIC EFFECTS OF ETHER AND OF ANTIBIOTICS ON A FECAL SUSPENSION

30 MIN. AT ROOM TEMPERATURE						
AMOUNT PER ML. FECAL SUSPENSION		CULTURES			CULTURES	
PENICILLIN (UNITS)	STREPTOMYCIN (MG.)	BLOOD AGAR	THIOGLYCOLATE BROTH	ETHERIZATION METHOD	BLOOD AGAR	THIOGLYCOLATE BROTH
100	2.0	0	0	15% ether	0	Gr.– rods
200	4.0	0	0	15% ether	0	Gr.– rods, Gr.+ cocci
300	6.0	0	0			
400	8.0	0	0			
500	10.0	0	0			
600	12.0	0	0			
700	14.0	0	0			
800	16.0	0	Few Gr.+ and Gr.– rods			
900	18.0	0	0			
1000	20.0	0	0			
None	None	0	Gr.– rods, Gr.+ rods, and cocci	No ether	0	Gr.– rods, Gr.+ rods and cocci

(2) Also, a comparison was made of the bacteriostatic effect of etherization with that of the antibiotics. A 20 per cent suspension of normal feces in sterile distilled water was centrifugated for ten minutes at 2,000 revolutions per minute to throw down the coarse particles. The supernatant fluid was removed and run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute. Cultures were made on blood agar and in thioglycolate broth. The material was divided and one part was treated with 15 per cent ether and the other with varying concentrations of the two antibiotics. The etherized portion was shaken for thirty minutes in the cold room and left overnight; the ether was removed by suction the next morning. Cultures were made in the media mentioned.

As may be noted in Table III, the ether attenuated the development of aerobic bacteria on blood agar but did not reduce growth in the anaerobic liquid medium. On the other hand, organisms were inhibited on both types of media when practically all of the antibiotic concentrations were used.

From the results both of titrations in monkeys and of the cultural tests, it seemed evident that the use of penicillin and streptomycin would be advantageous in the isolation of the poliomyelitis virus from fecal specimens. The optimal concentrations for bacterially infected material appeared to be 1,000 units of penicillin and 20 mg. of streptomycin per milliliter. These amounts retarded bacterial growth without reducing the virus potency. Therefore the method seemed sufficiently reliable for use on human fecal material.

Experiment 5. Use of the Antibiotics in the Preparation of Human Feces From Patients With Poliomyelitis.—

With Known Positive Feces.—Before trying this method on feces of unknown virus content, material was prepared from a human stool that had yielded poliomyelitis virus by the etherization method. This specimen had been kept frozen in the dry ice refrigerator for at least two months. A 20 per cent suspension in sterile distilled water was divided into two portions, one of which was treated with ether by the afore-mentioned method and the other with the antibiotics essentially in the manner described under Experiment 3, except for the following differences. The dilutions were 1:5, 1:10, 1:50, and 1:100, and 1,000 units of penicillin and 20 mg. of streptomycin were used per milliliter of dilution. Cynomolgus monkeys were inoculated throughout the experiment. The different dilutions of feces treated by the two methods were inoculated into the animals intraabdominally. The untreated suspensions were given intranasally.

TABLE IV. COMPARISON OF VIRUS ISOLATION METHODS IN CYNOMOLGUS MONKEYS USING A SUSPENSION OF POSITIVE POLIOMYELITIS FECES

METHOD	AMOUNT AND ROUTE OF INOCULATION*	RESULTS: DILUTIONS OF POSITIVE POLIOMYELITIS FECES			
		1:5	1:10	1:50	1:100
Etherization	30 ml. i.abd. 15 ml. i.n.	0	Paralyzed	0	0
Penicillin and streptomycin	30 ml. i.abd. 15 ml. i.n.	Paralyzed	Paralyzed	Paralyzed	0
Penicillin and streptomycin	1 ml. i.c.	Paralyzed	Paralyzed	0	0

Histopathologic sections on all paralyzed monkeys were positive for poliomyelitis.
*i.abd., Intra-abdominal; i.n., intranasal; i.c., intracerebral.

A third series of monkeys was injected intracerebrally with 1 ml. amounts of each dilution of feces treated with the antibiotics. As shown in Table IV, only the monkey given the 1:10 dilution of feces became paralyzed after the etherization method, while the animals given all three dilutions (1:5, 1:10, and 1:50) developed poliomyelitis after employing the antibiotic technique. No animals developed peritonitis nor did any show evidence of a brain abscess when inoculated intracerebrally. It seemed apparent that better results were obtained in the isolation of the virus by the antibiotic method than by that of etherization. The former was also simpler and quicker to prepare.

With Feces From Patients with Suspected Poliomyelitis.—Fecal specimens obtained during the first week of illness were received from eight patients with suspected poliomyelitis. Each sample was diluted in sufficient sterile distilled water to make a 20 per cent suspension, which was then centrifugated for ten minutes at 2,000 revolutions per minute to throw down the larger particles. The supernatant fluid was removed. About 15 ml. were saved and frozen in 3 ml. amounts to be used for the daily intranasal instillations of Cynomolgus monkeys. The remainder of the material was put in the angle centrifuge for thirty minutes at 13,000 revolutions per minute in the cold room (9 to 10° C.). The supernatant fluid was then kept frozen in 10 ml. amounts until needed.

Thirty minutes before the inoculations were to be made, 10 ml. of the suspension were thawed rapidly and treated with 1,000 units of buffered crystalline sodium penicillin G and 20 mg. of streptomycin (calcium chloride complex, Merck) per milliliter. After leaving the mixture for thirty minutes at room temperature, cultures were made on blood agar and in thioglycolate broth. A monkey was then inoculated intra-abdominally with 10 ml. of the treated material and intranasally with 3 ml. of the untreated feces. The same amounts were repeated daily over a five-day period until a total of 30 ml. was given into the peritoneal cavity and 15 ml. by the nasal route.

Four of the eight monkeys developed flaccid paralysis typical of poliomyelitis within from six to eight days after the first injection. They were sacrificed and suspensions of the cord were passed intracerebrally to monkeys, guinea pigs, and albino mice. The passage monkeys likewise developed symptoms of poliomyelitis, but the other animals remained well. Histopathologic sections of the first passage monkey cord showed lesions of poliomyelitis.

The results of these inoculations again indicated that the additions of penicillin and streptomycin in the quantities employed is a satisfactory aid in the isolation of the virus of poliomyelitis from bacterially contaminated suspensions.

DISCUSSION

Although the treating of fecal specimens with ether[2] in the isolation of the poliomyelitis virus has yielded adequate results in determining the causative agent in an outbreak of suspected poliomyelitis, there is always the risk of losing monkeys from peritonitis even if the bacterial counts seem negligible. If the peritoneal route is omitted and one depends solely on the intranasal instillations, the quantity of material administered may not be sufficient unless the time period is increased. Therefore it is gratifying to find that one can satisfac-

torily add penicillin and streptomycin in the afore-mentioned amounts to fecal or other bacterially contaminated material suspected of containing the virus of poliomyelitis.

In attempted isolation of this virus from feces obtained in an earlier outbreak, five out of twenty-two monkeys (22 per cent) died from peritonitis after intra-abdominal inoculation of etherized specimens. After inaugurating the antibiotic method, no animals developed peritonitis out of the twenty-five tested intra-abdominally, and none of the monkeys had a brain abscess after intracerebral injection of treated material. In applying this method, however, it is essential that the penicillin and streptomycin should be added to the fecal suspension after clarification in the angle centrifuge at 13,000 revolutions per minute. The virus is not sedimented at that speed and this type of machine may be available in most virus laboratories that do not have an ultracentrifuge. This method greatly reduces the time of preparation because the antibiotics react quickly and it is not necessary to leave the mixture in contact for more than thirty minutes.

As shown in the foregoing experiments (Tables I and IV), there is apparently no loss of virus potency because it may be recovered even after dilution of a fecal specimen 1:50, whereas the results were negative with this dilution when the same sample was treated with ether. There is sufficient evidence to show, therefore, that 1,000 units of penicillin and 20 mg. of streptomycin per milliliter do not materially inhibit the recovery of the poliomyelitis virus from contaminated material even though in a comparatively low concentration.

After this work was completed it was of interest to learn that Melnick[9] had been adding these antibiotics to human feces processed in the ultracentrifuge and that Corria[10] in Cuba also had been adding penicillin to fecal specimens with satisfactory results in the recovery of the poliomyelitis virus.

SUMMARY

It has been found that a mixture of 1,000 units of penicillin and 20 mg. of streptomycin per milliliter of material are the optimal concentrations to use for the inhibition of bacterial growth and the isolation of the virus of poliomyelitis from fecal specimens.

A method of isolating the virus from contaminated material has been described which has proved to be quicker and more efficient than the older etherization procedure.

Whereas previously five out of twenty-two (22.7 per cent) monkeys had developed peritonitis after intra-abdominal inoculation by the ether method, none of the twenty-five animals was lost after treatment of the suspensions with the antibiotics, and the same material could be used effectively by the intracerebral route as well.

The antibiotics did not prevent paralysis in monkeys when added to a 1:500 dilution of the MV strain, to a 1:1,000 dilution of MV virus and fly suspension, to a 1:10 dilution of MV virus and feces, or to a 1:50 dilution of a known positive fecal specimen.

With the new method, the virus of poliomyelitis was isolated from four out of eight (50 per cent) human feces taken during a recent outbreak of poliomyelitis.

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THE EFFECT OF COMMERCIAL HEPARIN ON THE PLATELET COUNT

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Copley and Robb[1] observed that when heparin was added to dog's blood in vitro, it failed to preserve the blood platelets. This was in contrast to the action of citrate which is presumed to preserve platelets because of its anticoagulant properties. The platelet count was lowered sharply within five minutes and after twenty-four hours showed reductions of from 30 to 100 per cent. Since the effect of heparin increased with its concentration and since Copley and Robb[2] also found that intravenous injections depressed the platelet count, there was indication of a direct action rather than a simple failure to preserve the platelets from unfavorable conditions ex vivo. This observation is of importance in considering the effect of heparin in the prevention of platelet thrombi. In the course of other studies on heparin, we have been able to confirm and extend the observations of Copley and Robb, and in a personal communication from Dr. A. J. Quick we learn that our results on dogs agree in general with some recent findings at his laboratory.*

METHODS

Platelet counts were made from blood taken into Rees and Ecker's formalin-citrate mixture recommended by Tocantins[3] and the technique of counting was in some cases modified by the use of 5 per cent urea solution as described by Fidlar and Waters[4] so that estimates of leucocytes could also be made in the same chamber in which the platelets were counted. Agglutination was estimated by counting clumps of three or more separately and expressing as a percentage of the total platelet count. In studying the influence of heparin on platelets outside the body, the silicone technique was used as described by Jaques, Fidlar, Feldsted, and Macdonald[5] in order to minimize the effect of foreign surfaces. Concentration of heparin† is expressed in Connaught anticoagulant units. This is the activity of 1/100 mg. of the crystalline barium salt or 1/110 mg. of the sodium salt of beef heparin.

RESULTS

In a series of experiments designed to study the physiology of heparin, various doses of the anticoagulant were injected into dogs. The effect of these injections on red cell, white cell, and platelet counts is shown in Table I. Attention is directed to the following points.

(1) A fall in the platelet count occurred in all instances and in four reached a level of less than 10 per cent of the number before injection.

(2) Fifty units per kilogram had as marked an effect in one animal as 1,500 units per kilogram had in another, suggesting either individual variability or no

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*See p. 1424.

†All the heparin was supplied by the Connaught Medical Research Laboratories, Toronto, Ont., except one sample of Danish heparin obtained through the kindness of Dr. Tage Astrup.

correlation between dosage and the extent of effect, provided the dose was beyond some minimum and given in a single injection.

(3) Agglutination of the platelets appeared in all samples taken two and one-half minutes to more than one hour after injection, but clumping under ten per cent was not regarded as significant.

(4) After single injections the count would recover from the initial fall, but continuous injection would hold the count down, although not at as low a level as followed the first injection.

(5) On the whole, the leucocytes declined and returned parallel with the platelets after single injections but remained at a lower level with continuous injection.

TABLE I. CELL COUNTS AFTER INTRAVENOUS INJECTION OF HEPARIN IN THE DOG

EXPERIMENT	HEPARIN (UNITS/KG.)	TIME	R.B.C./C.MM. × 1,000	W.B.C./C.MM. × 1,000	PLATELETS/C.MM. × 1,000	PLATELETS/C.MM. AGGLUTINATION (%)
22	500	Before	5,588	12.1	495	0
		3½ min.	5,594	12.2	135	56
3	500	Before	4,763	9.4	336	0
		2½ min.	4,956	8.0	143	59
		15 min.	4,781	2.6	148	12
5	500	Before	5,206	8.9	316	0
		15 min.	5,213	6.6	209	10
24	100	Before	5,794	10.0	295	1
		5 min.	5,781	5.2	20	24
		37 min.	5,981	9.1	228	5
25	1500	Before	5,369	8.5	341	0
		3½ min.	5,363	1.8	15	27
		60 min.	6,013	3.8	115	42
30	50	Before	4,225	9.7	295	0
	Then by	4 min.	3,756	3.9	21	67
	cont. inj.*	80 min.	4,231	3.1	107	60
7	50	Before	6,306	9.8	397	0
	Then by	4 min.	6,344	3.8	39	48
	cont. inj.*	65 min.	5,675	3.8	234	27
		407 min.	6,881	14.0	203	21
		17¾ hr. after cont. inj. ceased	6,400	25.0	191	0
9	30	Before	6,500	10.1	356	0
		4 min.	6,256	12.5	178	32
		30 min.	6,094	13.0	298	2
16-1A	1,000 u./kg.	Before	4,500		90	0
	injected	2 min.	4,500		50	0
	in 1 sec.	5 min.	4,700		50	20
		10 min.	4,500		50	50
		60 min.	4,600		85	0
16-2	1,000 u./kg.	Before	5,400		120	0
	injected	3 min.	5,000		30	0
	in 1 sec.	10 min.	5,000		115	0
		60 min.	5,400		130	0
16-1B	1,000 u./kg.	Before	5,000		142.5	0
	injected	2 min.	4,800		50	0
	in 1 min.	5 min.	5,000		55	0
		10 min.	5,100		60	0
		30 min.	4,500		85	0
		60 min.	5,100		120	0

*Continuous injection of 1 unit per kilogram per minute.
In the first eight experiments platelet counts were made in the 1/10 dilution with urea.
In the last three experiments platelet counts were made in the 1/100 dilution.

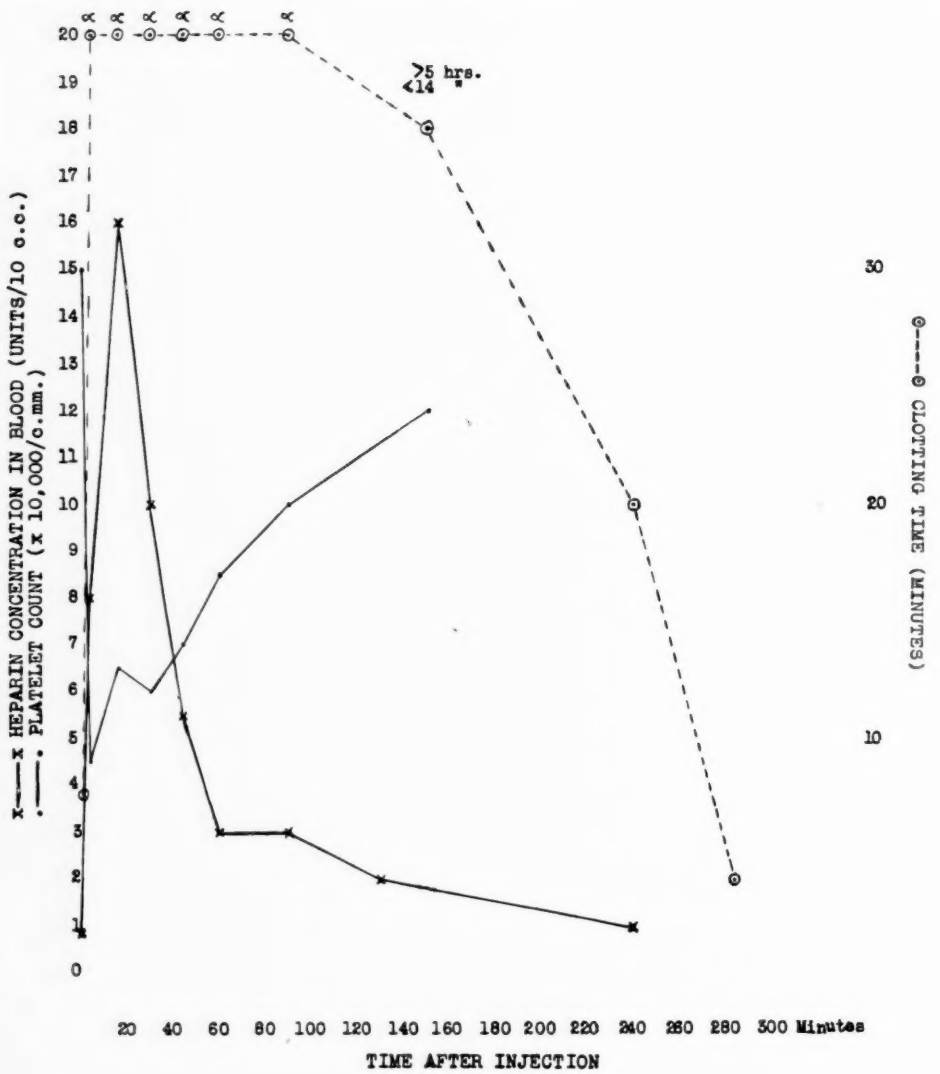
x——x HEPARIN CONCENTRATION IN BLOOD (UNITS/10 c.c.)
•——• PLATELET COUNT (x 10,000/c.mm.)
o——o CLOTTING TIME (MINUTES)
>5 hrs.
<14 "
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
10 20 30
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 Minutes
TIME AFTER INJECTION

Fig. 1.

The effects of continuous injection suggested that as long as heparin remained in the blood stream the counts would remain depressed. This was given further investigation in an experiment shown in Fig. 1 in which heparin concentration in the blood was determined by the method of Jaques, Monkhouse, and Stewart.[6] Four milligrams per kilogram of heparin were injected intravenously into a dog weighing 6.7 kilograms under pentobarbital anesthesia. Clotting times were determined by the method of Lee and White,[7] using blood from the exposed femoral vein. After injection of the heparin the platelet count fell to one-third of the initial value and then rose again, moving inversely with the heparin in the blood. It will be noted, however, that when the platelets recovered to 80 per cent of the original count, there was still enough heparin present

TABLE II. EFFECT OF INJECTED HEPARIN ON THE PLATELET COUNT IN L. B. J.

HEPARIN (LOT 1123)	TIME FROM INJECTION	R.B.C./C.MM. × 1,000	PLATELETS/C.MM. × 1,000
40 units/kg intravenously	Before		255
	45 sec.		190
	7 min.		155
	30 min.		255
40 units/kg. subcutaneously	Before	5,000	180
	5 min.	5,250	190
	1 hr.	4,900	120
	2½ hr.	4,900	90
	5 hr.	5,000	110
	23 hr.	5,050	150
	43 hr.	4,950	185

Counts made in the 1/100 dilution.

to prolong the clotting time to more than five hours. This extension of the anticoagulant action beyond the effect upon platelets will be discussed later.

Table II shows the effect of heparin upon one of us (L. B. J.) when administered both intravenously and subcutaneously. The latter route merely delayed and prolonged the thrombocytopenia.

Copley and Robb[1] showed that when mixtures of heparin and blood were stored in vitro, the greater part of the fall in the platelet count took place in the first forty or fifty minutes when compared with counts obtained at forty-eight hours. In the hope of securing further information we made observations on blood handled by means of the silicone technique. Under proper conditions and without the addition of any anticoagulant, it is sometimes possible by this method to obtain blood in which the platelet count remains practically unaltered for thirty minutes. Blood removed with a silicone syringe was transferred to small beakers coated with silicone and containing varying quantities of heparin. Since the controls with saline alone could not be used beyond thirty minutes, controls in citrate were used in some instances. For storage the mixture was poured into a silicone test tube, stoppered, and placed in the refrigerator. In a few experiments, as a further control, blood was also placed in a beaker without a silicone surface.

TABLE III. BLOOD AND HEPARIN MIXED IN SILICONE VESSELS

	PLATELET COUNTS IN FIRST SAMPLE AFTER MIXING			
	CONTROL MIXTURE		HEPARIN MIXTURE	
SUBJECT		PLATELETS/C.MM. × 1,000		UNIT/C.C.
Man	Saline	220	140	10
	Saline	190	125	10
	Saline	135	120	100
Dog	Citrate	312	220	0.1
	Citrate	312	319	1.0
	Citrate	312	218	10
	Citrate	312	232	100
	Citrate	310	284	10
	Citrate	310	294	100
	Citrate	306	256	10
	Citrate	306	261	100
	Tyrode's	294	266	100
	Saline	120	65	10
	Saline	115	55	10
	Saline	195	160	10
	Saline	100	80	10
	Saline	185	120	10
	Saline	140	90	10

Table III gives a summary of samples taken as soon as possible after mixture in silicone vessels. The time of sampling would vary from a few seconds to about three minutes depending upon the manipulations required in different experiments. In only three of the fifteen examples did the count of the heparin mixture correspond with that of the control mixture within the error of the method, and in two of these it was lower than the value for the control. This demonstrates the promptness of the heparin's effect.

TABLE IV. EFFECT ON THE PLATELET COUNT OF MIXING HEPARIN AND BLOOD IN SILICONE VESSELS

HEPARIN AND HUMAN BLOOD IN SILICONE VESSELS						
EXPERIMENT A		EXPERIMENT B			EXPERIMENT C	
HEPARIN (10 U./C.C.)		HEPARIN (10 U./C.C.)		SALINE	HEPARIN (100 U./C.C.)	
TIME OF SAMPLE	PLATELETS × 1,000	TIME OF SAMPLE	PLATELETS × 1,000	PLATELETS × 1,000	TIME OF SAMPLE	PLATELETS × 1,000
Immed.	125 (4%)*	Immed.	140	220	Immed.	120
3 min.	105 (5%)	3 min.	100	225	7 min.	70
10 min.	120 (18%)	7 min.	60	165	15 min.	75
16 min.	80 (12%)	15 min.	85	255	24 hr.	50 (30%)
23 hr.	110 (22%)	30 min.	85	130	48 hr.	70
		24 hr.	70	Clotted		

HEPARIN AND CANINE BLOOD IN SILICONE AND PLAIN GLASS VESSELS						
	SILICONE	SILICONE	SILICONE	SILICONE	SILICONE	PLAIN GLASS
	HEPARIN (0.1 U./C.C.)	HEPARIN (0.1 U./C.C.)	HEPARIN (10 U./C.C.)	HEPARIN (100 U./C.C.)	CITRATE CONTROL	HEPARIN (10 U./C.C.)
TIME OF SAMPLE	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000
Experiment D						
Immed.	220	319	218 (3%)	232 (2%)	312	276 (7%)
9½ min.	192 (15%)	249 (21%)	204 (38%)	190 (16%)	325 (5%)	239 (51%)
30 min.	225 (11%)	326 (51%)	150 (80%)	146 (76%)	184 (4%)	81 (69%)
2½ hr.	7 (42%)	193 (39%)	97 (54%)	59 (84%)	231 (24%)	170 (66%)
24 hr.	Clotted	62 (9%)	152 (30%)	99 (57%)	256 (12%)	122 (25%)
48 hr.		Clotted	189 (19%)	122 (62%)	205 (5%)	143 (14%)
72 hr.			210 (14%)	200 (41%)	221	137 (28%)
1 wk.			108 (14%)	87 (29%)	304	150 (34%)
Experiment E						
Immed.			284	294	310	259
10 min.			191 (74%)	103 (85%)	210 (26%)	252 (61%)
30 min.			129 (67%)	55 (80%)	178 (51%)	149 (73%)
3 hr.			128 (51%)	93 (83%)	174 (46%)	133 (60%)
24 hr.			27 (27%)	73 (27%)	200 (24%)	44 (23%)
48 hr.			60 (12%)	104 (32%)	187 (24%)	101 (17%)
72 hr.			41 (17%)	95 (17%)	193 (13%)	68 (21%)
Experiment F						
Immed.			256 (77%)	261 (71%)	325 (3%)	295 (67%)
12 min.			109 (93%)	250 (93%)	266 (20%)	101 (90%)
33 min.			126 (85%)	109 (91%)	239 (25%)	215 (88%)
3 hr.			159 (43%)	199 (86%)	295 (8%)	259 (50%)
24 hr.			219 (40%)	195 (37%)	279 (4%)	180 (22%)
Experiment G (Saline in Place of Citrate Control)						
Immed.			65 (4%)		120	
3 min.			40		100	
7 min.			45 (33%)		110	
15 min.			120 (88%)		Clotted	
30 min.			130 (77%)			
Experiment H (Tyrode's Solution in Place of Citrate Control)						
Immed.				266	294	
6 min.				229 (49%)	195	
23 min.				108 (75%)		

*Percentages in brackets are agglutination.

In Experiments A, B, C, and G platelet counts were made in the 1/100 dilution.

In Experiments D, E, F, and H platelet counts were made in the 1/10 dilution with urea.

Table IV gives the results obtained in later samples and attention is directed to the following points.

(1) The decline initiated at mixing was continued to a low point depending on the time of sampling but varying from three minutes to twenty-four hours.

(2) In some instances a recovery occurred from this low point which could hardly be accounted for on the basis of irregular sampling.

(3) Citrate controls observed for twenty-four hours or more in three experiments showed a similar decline and recovery but less marked. Even a control in saline would sometimes show this within the half hour, although the red and white cell counts were within the error of the method.

(4) As in the experiments in vivo there appeared to be an individual variability.

These findings raise several points of interest which will be discussed later.

TABLE V. PLATELET COUNTS AFTER HEPARIN IN VIVO AND WHEN HEPARINIZED BLOOD IS ADDED TO FRESH HEPARIN (10 UNITS/C.C.) IN SILICONE VESSELS

TIME AFTER MIXING		SALINE CONTROL	IN SILICONE VESSEL	AGGLUTINATION
	Experiment 16-2			
			Count in silicone	
–	Sample for silicone	115,000	55,000	
3 min.			65,000	
7 min.			25,000	
15 min.			85,000	
30 min.			50,000	
Heparin injected intravenously 1,000 units/kg.			Count in vivo	
–			120,000	
3 min.			30,000	
10 min.			115,000	
60 min.			130,000	
–	Sample for silicone		Count in silicone	
–		135,000	135,000	
3 min.		130,000	135,000	5%
7 min.		125,000	80,000	
10 min.		135,000	95,000	
30 min.		100,000	115,000	30%
	Experiment 16-5			
	Sample for silicone		Count in silicone	
–		195,000	160,000	
5 min.			80,000	
15 min.			55,000	
30 min.			120,000	
Heparin injected intravenously 10 units/c.c. of blood			Count in vivo	
–			220,000	
10 min.			50,000	
60 min.			195,000	
Heparin injected intravenously				
–			185,000	10%
10 min.			60,000	25%
60 min.			150,000	13%
Heparin injected intravenously 10 units/c.c. 15 min. later				
–			150,000	
10 min.			90,000	
60 min.			140,000	
	Sample for silicone		Count in silicone	
–		150,000	150,000	
5 min.		120,000	90,000	
15 min.		150,000	80,000	28%
20 min.		150,000	85,000	

Platelet counts made in the 1/100 dilution.

As a further test of the effect of heparin on platelets, a comparison was made of the counts in vivo with counts on the same blood *ex vivo*. Using the silicone technique, blood from a dog was added to heparin in a beaker and then heparin was injected into the same animal to bring the concentration in the circulating blood to approximately that in the beaker. An hour later another sample of blood was withdrawn and added to heparin in a silicone beaker. The results for two experiments are shown in Table V. Here it will be noted that:

(1) The reduction that occurred immediately after mixing the first sample in the silicone vessel continued to a low point at seven minutes and reached a level approximating that found three minutes after injection into the animal.

(2) Blood drawn from the heparinized animal did not show the usual reduction in platelets immediately after mixing with fresh heparin in the beaker.

(3) In the second experiment three successive injections were each followed by a sharp decline in the platelets although at the time of the last two injections the blood was incoagulable.

(4) The counts in the second animal at the time of each injection were at successively lower levels and declined less with each successive injection.

Platelet counts were made by one of us (E. F.) in connection with experiments on heparin at Toronto in 1938-1941. On one occasion samples of blood which were taken into heparin instead of formalin-citrate gave unexpectedly low counts together with clumping. As a consequence, this technique was never repeated. On the other hand, there were experiments in which samples of blood which had been taken ten or fifteen minutes after injections of heparin showed no significant drop, although in the light of present experience some reduction would have been expected. These facts, together with some differences between our results and those of Wright,[8] to be discussed later, raised the question of differences in samples of heparin and the possibility of an impurity in the commercial product we were using. No evidence of the latter possibility was found in fractional precipitation with brucine. There was no concentration of the agglutinating activity in any one fraction. After discussing the earlier experiences with Dr. A. M. Fisher* and Dr. A. F. Charles,* they very kindly provided certain experimental lots for comparison with the commercial product. Also through the kind cooperation of Dr. Tage Astrup a sample of Heparin Leo was obtained in powder form and was compared with a similar product from Dr. Charles. The results of this study are contained in Table VI.

Heparin I, the commercial sample, was already in solution in 10 c.c. vials with 0.3 per cent tricresol as preservative. Samples II to VI were experimental lots, but II differed from III only by the absence of tricresol. Samples VII and VIII were freshly dissolved in 0.9 per cent saline with 0.3 per cent tricresol and were used within an hour after solution. Sample VII assayed 110 units per milligram and sample VIII, the Heparin Leo, 100 units per milligram. All samples were given intravenously to dogs under Nembutal in a dose of 100 units per kilogram, and platelet counts were made before injection and at approximately four, ten, thirty, and sixty minutes afterwards. The figures are adjusted to the same basis relative to the erythrocytes. The following features are apparent in Table VI.

*Connaught Laboratories.

(1) One animal, C, was distinctly more sensitive than the other three.

(2) With one exception, the maximum agglutination coincided with the lowest count.

(3) With one exception, the lowest count was obtained in the first sample after injection.

TABLE VI. PLATELET AND LEUCOCYTE COUNTS WITH DIFFERENT SAMPLES OF HEPARIN GIVEN INTRAVENOUSLY TO DOGS

SAMPLE OF HEPARIN	ANIMAL A			ANIMAL B			ANIMAL C			ANIMAL D		
	PLATELETS			PLATELETS			PLATELETS			PLATELETS		
	× 1,000	AGGLUTINATION (%)	W.B.C. × 1,000	× 1,000	AGGLUTINATION (%)	W.B.C. × 1,000	× 1,000	AGGLUTINATION (%)	W.B.C. × 1,000	× 1,000	AGGLUTINATION (%)	W.B.C. × 1,000
I	334	0	7.7	275	0	11.1	261	0	9.8	252	0	5.3
	22	67	1.5	45	77	3.3	22	80	1.7	20	55	1.7
	59	54	6.9	49	55	6.6(a)	21	33	5.6	62	63	3.6
	274	12	6.2	260	27	8.8	140	35	7.0	175	25	3.6
	348	0	5.4	273	2	8.0	268	12	6.7	249	5	5.3
II	216	5	9.5				236	3	11.6			
	191	23	10.8(b)				105	29	13.2			
	204	2	8.3				195	1	13.0(c)			
	268	0	10.3				296	0	12.8			
	270	0	8.0				322	2	12.7			
III	225	4	10.6				230	8	9.7			
	186	19	10.5				133	26	9.3			
	253	1	10.3				16.	3	9.5			
	246	0	9.6				267	2	9.8			
	265	0	9.1				277	0	10.9			
IV	242	5	8.5				230	5	10.5			
	171	16	9.3				115	46	9.4			
	252	3	9.6				206	24	9.1(d)			
	244	2	7.1				248	0	7.4			
	2 9	3	6.1				229	0	6.2(e)			
V	187	8	7.4				208	6	10.9			
	125	15	9.5				84	54	10.8			
	158	3	6.7				137	21	11.5			
	201	2	6.3				258	0	12.3			
	183	8	6.1				236	0	11.8			
VI	194	1	7.1				234	0	10.6			
	182	13	7.9				126	23	12.5			
	241	0	8.7				186	1	11.0			
	231	0	6.3				299	0	13.9			
	240	0	4.5				314	0	13.1			
VII							241	0	10.4	362	0	12.8
							47	29	11.4	190	49	11.3
							91	17	10.7	276	8	9.1
							287	1	13.2(f)	394	2	11.7
							314	0	12.2	362	0	9.4
VIII							225	0	8.9	349	0	12.4
							42	30	9.1	86	63	12.2
							75	18	9.1	260	14	10.8
							244	1	10.7	360	0	11.7
							300	0	11.2	390	1	8.4

Blood samples taken before injection and at four, ten, thirty, and sixty minutes after except as follows: (a), at eleven and one-half minutes; (b), at three and one-half minutes; (c), at eleven minutes; (d), at sixteen and one-half minutes; (e), at sixty-two and one-half minutes; (f), at thirty and one-half minutes.

Blood diluted 1/5 in formalin-citrate. This 1:5 mixture diluted 1/10 with 5 per cent urea solution for counts.

Heparin dosage 100 units per kilogram.

(4) The sharpest reaction occurred with the commercial sample of heparin and was accompanied by a definite leucopenia.

(5) Some experimental products given in the same dosage of anticoagulant units as the commercial one had much less effect upon platelets.

DISCUSSION

These results agree with those of Copley and Robb[2] that the injection of commercial heparin into dogs will cause a transient thrombocytopenia. This result also has been found in man, as one of the authors (L. B. J.) demonstrated upon himself. Copley[9] has found platelet emboli in the rabbit and hamster following injection of heparin. Hence the phenomenon appears to be a general one, although in mice Copley and Robb[2] did not demonstrate the platelet change as clearly as in dogs. In the latter they found the maximum thrombocytopenia to be about 40 per cent, whereas in seven of our dogs the decrease reached 90 per cent or more. This may be due to the fact that their samples were not taken until twenty-six or more minutes after injection, although in three of their animals studied at shorter intervals the lowest platelet count occurred in the first twenty minutes.

The lowest counts in our experiments appeared within three and one-half to five minutes after intravenous injection. A slow rate of injection, 1,000 units per kilogram per minute, was practically as effective as a rapid rate, 1,000 units per kilogram per second. In the human subject, subcutaneous administration appeared to delay and prolong the effect of heparin but not to abolish it. Continuous injection at 1 unit per kilogram per minute held the platelet count down, though not at the low point reached by a single injection. During the period when the blood was incoagulable from one injection of heparin, a second injection would again depress the count. In the first experiment, in Table V, the dose of 1,000 units per kilogram would prolong the clotting time to about eight hours, but the count in this animal returned to normal in one hour. Compared with the experiment shown in Fig. 1, the return of the count was more rapid in spite of the larger dose. This is another example of the individual variability encountered among dogs, but both instances favour the idea that some substance may render the heparin innocuous to platelets without inhibiting the anticoagulant power, or that the agglutinating action is a weaker property. It is interesting that the heparinized blood, drawn from the animal in Table V, when added to fresh heparin in the silicone beaker did not show the immediate drop in platelets commonly found with normal blood. This might mean greater resistance acquired by the platelet or perhaps the disappearance of sensitive platelets due to the injected heparin.

On the question whether the extent of the decline in vivo depends upon dosage, our data do not suggest any correlation. In Table I it will be seen that there was practically no difference in the extent of response to 50 units per kilogram in one animal and 1,500 units per kilogram in another, although in the latter instance there is evidence of a more prolonged effect if compared with the preceding experiment where 100 units per kilogram were used. In both Copley and Robb's[2] results and our own the differences point to individual variability.

In vitro, however, where the same sample of blood can be used with varying doses, Copley and Robb[7] showed that in strengths from 0.05 to 100 units per cubic centimeter the platelet count decreased as the heparin increased. Also they noted that this effect of heparin did not appear when it was added to citrated blood.

The mechanism of the swift disappearance and return of the platelets from the general circulation is not clear. The appearance of clumps of platelets in blood samples taken soon after the injection suggests that a filtering out of clumps in the capillaries is the cause of the sharp fall in vivo. But actual lysis may also be a possibility, and the increase in adhesiveness leading to agglutination may be merely an early stage of lysis. Wright[8] in her study of adhesiveness noted only about a 10 per cent decline in the count in eighty minutes when using heparin with blood in paraffined tubes. It is admitted that our method of estimating agglutination is only a rough approximation, but if the data of Experiments D, E, and F, Table IV, are examined it will be noted that the maximum agglutination occurred in the ten- or thirty-minute samples. Where the heparin used was 10 units per cubic centimeter or more, considering samples only up to three hours, the maximum agglutination either coincided with the lowest count or appeared in the sample immediately preceding it. All samples of twenty-four hours or later showed a decline in agglutination from the maximum. This corresponded with a rising tendency in the counts in two of the blood specimens (Experiments D and F), though in Experiment E the counts tended to remain low. This inclines us to the opinion that clumps can break up again, especially in the living animal where there may be some factor tending to neutralize the heparin effect as suggested in Table V. On the other hand, we have observed in late samples large clumps in which it was impossible to count individual platelets and which gave the suggestion of coalescence as though undergoing a slow form of lysis.

Lysis is also suggested in the mixtures in silicone because samples taken simultaneously from the center, bottom, and side of the beaker gave counts which were all within experimental error. For example, in one experiment the count from the center of the beaker was 100,000 from saline, 80,000 after three minutes in heparin, and 45,000 after thirty minutes in heparin. Other samples taken at the same time from side and bottom were within plus-minus 5,000 per cubic millimeter. If the decrease in the count were due to settling of clumps or their adhesion to the walls of the vessel one would expect a greater variation.

Against the idea of lysis of the platelets as a major factor in the decline are, first, the very rapid recovery and, second, the absence of any symptoms which might be expected from materials known to be present in platelets (thromboplastin, vasoconstrictor substances, and so on).

The rapid return of the platelet count in some of the experiments in vivo was remarkable. In some cases after declining 60 to 75 per cent it reached the normal level again within an hour. This scarcely can be due to new formation of platelets. Lawrence and Valentine[10] estimated production in the cat to be about 1,600 to 2,800 per cubic millimeter per hour. Tocantins[11] found 40,000 per cubic millimeter per hour following splenectomy, but this rate could hardly

be expected from normal bone marrow. A count rising from 20,000 to 228,000 in thirty-two minutes (Table I) does not look like new formation. It may be noted also that since the count before heparin was 295,000 no great destruction by lysis is indicated.

The fact that the leucocytes tend to decline when the platelet count is very low suggests that they also are caught with the platelet clumps in the capillaries. In one experiment, freshly drawn blood was mixed with heparin in the syringe, placed in a plain glass test tube, and kept fifteen minutes at body temperature before reinjection. After the blood had been drawn back into the syringe, it was found that the wall of the tube had a deposit of fine granules which, when washed off in formalin-citrate, proved to be platelet clumps. Many of these were adherent to leucocytes.

It is unlikely that there is any direct destructive action of heparin on the leucocytes since counts of the latter were made on all samples in Experiments D, E, and F (Table IV) and failed to show the parallelism with the platelet counts usually found in vivo.

The silicone experiments require further consideration. They were planned as controls of the in vivo experiments on the assumption that since contact with glass could be avoided, the conditions outside the body would resemble those within. The data did not support this assumption. The citrate controls in silicone listed in Table IV showed a 41 and 44 per cent reduction in two cases and 27 per cent in the third where the citrate was raised to 3.8 per cent of the final mixture. In another experiment not shown, an oxalate control in plain glass gave a 40 per cent reduction. Where heparin was the only anticoagulant present at 10 units or more per cubic centimeter, the platelet fall in silicone ranged from 56 to 75 per cent in Experiment D, 81 to 90 per cent in Experiment E, and 57 to 66 per cent in Experiment F, so that the decline in all three was definitely greater than with citrate or oxalate. A plain glass surface may be considered a uniform stimulus for platelet change, and in tests with heparin Wright found a reduction of 55 per cent to over 70 per cent compared with 10 per cent in the paraffin control. We found it rather surprising, therefore, that there was not sufficient difference between plain glass and silicone vessels to justify the thought that the character of the surface is an important factor in the reduction of platelets when anticoagulants are present. This result, together with other experiences with silicone, leads to the suggestion that at the moment of mixing certain changes affecting the platelet already may have been initiated and are continued even though at a retarded rate and to a limited extent after the addition of anticoagulants.

It should be pointed out that good blood samples for the silicone technique are more difficult to obtain from dogs than from human subjects. This is emphasized by the careful experiments of Patton, Ware, and Seegers.[12] Since Wright's tests were made with human blood, the differences mentioned between her results and ours may be related to this fact. Another variable which may be involved is suggested by the work of Brinkhous[13] who has presented evidence of a plasma factor necessary for the lysis of platelets; it is not difficult to imagine agglutination as a related phenomenon. In experiments on dogs receiving

Dicumarol but not recorded here, we have noted marked differences in the response of platelets from different animals to the same dose of heparin in vitro. It is evident, therefore, that these experiments with silicone cannot be regarded as simulating conditions within the blood vessel. Besides the changes in the blood incident to its removal, such factors as mixing and carbon dioxide tension are quite different in vitro compared with in vivo.

One of the puzzling features of the ex vivo experiments, illustrated in Table IV, was the return of the platelets after an initial decline when the blood mixtures were followed for twenty-four hours or longer. This did not occur in all blood samples as, for example, in Experiment E where the count remained consistently low from twenty-four to seventy-two hours. Even over shorter periods of time late samples might show increases over earlier ones, as in Experiment G, and occasionally even a saline control in silicone might show it, as in Experiment B. Undoubtedly this was partly due to uneven distribution, but the counts of red and white cells made at the same time did not show such wide variations. The decline in agglutination suggested one reason for an increase, but the platelets in the aging blood could not always be recognized as old and indeed one had to consider again whether platelets can be formed from red cells.

In Schilling's "ideal" red cell, a review of which is given by Ulpts,[14] two structures are indicated which easily may be counted as platelets, and indeed one of them was so labelled by Schilling. Occasionally the ghost of a red cell surrounding such a structure will enable one to eliminate it from the count, but it is possible that the inclusion of some of these Schilling bodies may be partly responsible for the rise of the count in late samples. But there cannot be a great many of these "ideal" erythrocytes because platelet counts made by the use of 5 per cent urea solution which hemolyzes red cells showed general agreement with platelet counts made in the usual formalin-citrate mixture, being sometimes lower and sometimes higher. Since the use of urea allows a dilution of only $\frac{1}{10}$ of the blood sample while the other count is made in the $\frac{1}{100}$ dilution, there was a much greater chance of the former showing an increase if many erythrocytes containing Schilling bodies were present. Rees and Ecker[15] called these refractile structures Arnold bodies, and the addition of formalin to the 3.8 per cent sodium citrate which they recommended made the laking of red cells less frequent. Irregularities in platelet counts made in the $\frac{1}{100}$ dilution suggest that the red cells may interfere with a free distribution of platelets *when clumps are present* and the error is multiplied when compared with the lower dilution. For this reason reliance has been placed on the count made in the urea solution and the values given in Table VI were so obtained.

With regard to Table VI it should be pointed out that the barbiturate may have some slight influence on the figures. The effect of this type of anesthesia in lowering the erythrocyte count is well known. During 1941-1942 in some experiments that were being made at Toronto by Dr. R. E. Haist on surgical shock, one of us (E. F.) noted the usual reduction in red cells following the barbiturate but found the platelets were not reduced in the same proportion. This has the effect of a relative increase and is quite definite when compared with preanesthetic counts. There was an indication that a slight relative increase might continue for

be expected from normal bone marrow. A count rising from 20,000 to 228,000 in thirty-two minutes (Table I) does not look like new formation. It may be noted also that since the count before heparin was 295,000 no great destruction by lysis is indicated.

The fact that the leucocytes tend to decline when the platelet count is very low suggests that they also are caught with the platelet clumps in the capillaries. In one experiment, freshly drawn blood was mixed with heparin in the syringe, placed in a plain glass test tube, and kept fifteen minutes at body temperature before reinjection. After the blood had been drawn back into the syringe, it was found that the wall of the tube had a deposit of fine granules which, when washed off in formalin-citrate, proved to be platelet clumps. Many of these were adherent to leucocytes.

It is unlikely that there is any direct destructive action of heparin on the leucocytes since counts of the latter were made on all samples in Experiments D, E, and F (Table IV) and failed to show the parallelism with the platelet counts usually found in vivo.

The silicone experiments require further consideration. They were planned as controls of the in vivo experiments on the assumption that since contact with glass could be avoided, the conditions outside the body would resemble those within. The data did not support this assumption. The citrate controls in silicone listed in Table IV showed a 41 and 44 per cent reduction in two cases and 27 per cent in the third where the citrate was raised to 3.8 per cent of the final mixture. In another experiment not shown, an oxalate control in plain glass gave a 40 per cent reduction. Where heparin was the only anticoagulant present at 10 units or more per cubic centimeter, the platelet fall in silicone ranged from 56 to 75 per cent in Experiment D, 81 to 90 per cent in Experiment E, and 57 to 66 per cent in Experiment F, so that the decline in all three was definitely greater than with citrate or oxalate. A plain glass surface may be considered a uniform stimulus for platelet change, and in tests with heparin Wright found a reduction of 55 per cent to over 70 per cent compared with 10 per cent in the paraffin control. We found it rather surprising, therefore, that there was not sufficient difference between plain glass and silicone vessels to justify the thought that the character of the surface is an important factor in the reduction of platelets when anticoagulants are present. This result, together with other experiences with silicone, leads to the suggestion that at the moment of mixing certain changes affecting the platelet already may have been initiated and are continued even though at a retarded rate and to a limited extent after the addition of anticoagulants.

It should be pointed out that good blood samples for the silicone technique are more difficult to obtain from dogs than from human subjects. This is emphasized by the careful experiments of Patton, Ware, and Seegers.[12] Since Wright's tests were made with human blood, the differences mentioned between her results and ours may be related to this fact. Another variable which may be involved is suggested by the work of Brinkhous[13] who has presented evidence of a plasma factor necessary for the lysis of platelets; it is not difficult to imagine agglutination as a related phenomenon. In experiments on dogs receiving

Dicumarol but not recorded here, we have noted marked differences in the response of platelets from different animals to the same dose of heparin in vitro. It is evident, therefore, that these experiments with silicone cannot be regarded as simulating conditions within the blood vessel. Besides the changes in the blood incident to its removal, such factors as mixing and carbon dioxide tension are quite different in vitro compared with in vivo.

One of the puzzling features of the ex vivo experiments, illustrated in Table IV, was the return of the platelets after an initial decline when the blood mixtures were followed for twenty-four hours or longer. This did not occur in all blood samples as, for example, in Experiment E where the count remained consistently low from twenty-four to seventy-two hours. Even over shorter periods of time late samples might show increases over earlier ones, as in Experiment G, and occasionally even a saline control in silicone might show it, as in Experiment B. Undoubtedly this was partly due to uneven distribution, but the counts of red and white cells made at the same time did not show such wide variations. The decline in agglutination suggested one reason for an increase, but the platelets in the aging blood could not always be recognized as old and indeed one had to consider again whether platelets can be formed from red cells.

In Schilling's "ideal" red cell, a review of which is given by Ulpts,[14] two structures are indicated which easily may be counted as platelets, and indeed one of them was so labelled by Schilling. Occasionally the ghost of a red cell surrounding such a structure will enable one to eliminate it from the count, but it is possible that the inclusion of some of these Schilling bodies may be partly responsible for the rise of the count in late samples. But there cannot be a great many of these "ideal" erythrocytes because platelet counts made by the use of 5 per cent urea solution which hemolyzes red cells showed general agreement with platelet counts made in the usual formalin-citrate mixture, being sometimes lower and sometimes higher. Since the use of urea allows a dilution of only $\frac{1}{10}$ of the blood sample while the other count is made in the $\frac{1}{100}$ dilution, there was a much greater chance of the former showing an increase if many erythrocytes containing Schilling bodies were present. Rees and Ecker[15] called these refractile structures Arnold bodies, and the addition of formalin to the 3.8 per cent sodium citrate which they recommended made the laking of red cells less frequent. Irregularities in platelet counts made in the $\frac{1}{100}$ dilution suggest that the red cells may interfere with a free distribution of platelets *when clumps are present* and the error is multiplied when compared with the lower dilution. For this reason reliance has been placed on the count made in the urea solution and the values given in Table VI were so obtained.

With regard to Table VI it should be pointed out that the barbiturate may have some slight influence on the figures. The effect of this type of anesthesia in lowering the erythrocyte count is well known. During 1941-1942 in some experiments that were being made at Toronto by Dr. R. E. Haist on surgical shock, one of us (E. F.) noted the usual reduction in red cells following the barbiturate but found the platelets were not reduced in the same proportion. This has the effect of a relative increase and is quite definite when compared with preanesthetic counts. There was an indication that a slight relative increase might continue for

a time in successive samples after anesthesia. This may be the reason why in Table VI some of the experiments show at thirty or sixty minutes a higher count than the initial one. We mention this as a caution in case this increase be interpreted as increased production in immediate response to the heparin. The anesthetic avoids the excitement incident to the taking of each sample in untrained dogs, and since the important count is within five minutes after heparin and is usually only a few minutes after the initial count, conditions appear more favourable for a comparison between different samples of heparin.

Platelets are sensitive to many foreign materials and it should be recalled that the synthetic anticoagulants studied by Astrup and Piper[16] also caused platelet agglutination, but the fact that heparin brings about this phenomenon has the appearance of a physiologic contradiction. The formation of platelet emboli does not seem to harmonize with the prevention of platelet thrombi. However, the very act of purification may cause commercial heparin to differ from that released within the animal body. The results shown in Table VI suggest that more work is required before platelet agglutination can be accepted as a property of natural heparin.

The effect of commercial heparin on platelets resembles its effect on chylomicrons seen in studies of lipemia by Waldron and Friedman.[17] It is possible that there is some common underlying factor for both these actions.

SUMMARY

Heparin on intravenous injection in man or dog may cause a brief thrombocytopenia. In dogs this may reach 10 per cent of the original count, although there is individual variability. The fall in the platelet count is repeated with repeated injections. The decline is also observed when heparin is added to blood in silicone vessels. Some experimental lots of heparin in the same dosage of anticoagulant units have shown much less effect upon platelets than the present commercial product.

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THE EFFECT OF HEPARIN ON PLATELETS IN VIVO

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WHEN either peptone or anaphylactic shock is produced in dogs, the platelets are temporarily but precipitously diminished, heparin is liberated into the blood, and histamine or a histamine-like substance is produced.[1] This triad has become of particular interest since Allen and Jacobson[2] have found that dogs exposed to highly ionized irradiation suffer a marked thrombocytopenia, heparinemia, and a peripheral capillary dysfunction manifested by petechiae. In a recent note Copley[3] implies that the heparin is responsible for the thrombocytopenia and that it "may also take part in the formation of the petechial hemorrhages." He cites his work and that of his associates in which it was shown that heparin causes an agglutination in vitro[4] and likewise an agglutination and subsequent thrombocytopenia in vivo.[5] As the result of these observations it becomes important to determine whether heparinemia produced either endogenously or exogenously causes thrombocytopenia, or whether the fall in platelets and the outpouring of heparin in shock and after high ionizing irradiation are concomitant occurrences resulting perhaps from a common agent. As a preliminary approach to this problem, the effect of intravenously administered heparin on the number of circulating platelets was studied.§

EXPERIMENTAL

The action of heparin on the platelet count was determined in rabbits, dogs, and men. The heparin employed was obtained from several pharmaceutical companies in the form of 10 c.c. vials each containing 100 mg. of the sodium salt of heparin. Several of these preparations have their potency stated in terms of Toronto units (1 mg. of the sodium salt of heparin is equivalent to 110 units).‖

The heparin was injected intravenously at a constant slow rate of approximately 1 to 2 c.c. per minute. In man only one standard dose was studied, namely 0.8 mg. (88 Toronto units) per kilogram of body weight, since this approximates the one generally employed for prophylaxis against thrombosis. In dogs and rabbits the doses ranged from 0.5 to 5 mg. (55 to 550 units) per kilogram of body weight. Samples of blood were taken before and at fixed intervals after the heparin was given. The vein into which the heparin was injected was not

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§After this investigation was completed, we were informed by Dr. Charles H. Best that Dr. Edward Fidlar and Dr. Louis Jaques had made a similar study. Dr. Fidlar kindly supplied us with a summary of their findings which are similar to ours. They too observed the rapid agglutination of platelets in dogs following the injection of heparin. See p. 1410.

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used for subsequent collection of blood. A silicone-coated syringe was used and the blood for the platelet count was transferred immediately to a silicone-coated test tube immersed in ice water. An alternative method consisted of placing 0.2 c.c. of 3.8 per cent sodium citrate directly in a 1 c.c. syringe and drawing blood exactly to the mark. The platelets were determined by direct count. The blood was diluted 1:200 with 3.8 per cent sodium citrate containing 0.2 c.c. formaldehyde (40 per cent) per 100 cubic centimeters.

In man the change in heparin concentration can be successfully followed by the coagulation time (Lee-White), the prothrombin time, and the thrombin titration.[6] In rabbits and dogs only the last method is satisfactory since the prothrombin time is too little affected, and the coagulation time is so markedly prolonged that it becomes impractical to perform and it is difficult to achieve accuracy.

RESULTS

The findings obtained by injecting heparin into man are given in Table I. It will be observed that the platelet count remained unaltered and that all of the heparin was removed from the blood in three to four hours. The bleeding time remained normal even during the time the heparin concentration in the blood was highest.

TABLE I. THE EFFECT OF INJECTING HEPARIN INTRAVENOUSLY IN MAN ON THE PLATELET COUNT, COAGULATION TIME, PROTHROMBIN TIME, AND THROMBIN TITRATION

	SUBJECT J. N. S. (WEIGHT): 80 KG.) HEPARIN: 64 MG.*				SUBJECT M. S. (WEIGHT: 63 KG.) HEPARIN: 50 MG.*			
TIME	PLATELETS (THOUSANDS)	COAGULATION TIME (LEE-WHITE) (MIN.)	PROTHROMBIN TIME (SEC.)	THROMBIN TITRATION ($\frac{1}{5}$ S.)† (SEC.)	PLATELETS (THOUSANDS)	COAGULATION TIME (LEE-WHITE) (MIN.)	PROTHROMBIN TIME (SEC.)	THROMBIN TITRATION ($\frac{1}{5}$ S.)† (SEC.)
0	186	5¾	12	7	116	6	12	6.5
5 min.	168	60	27	255	122	42	22	125
15 min.	180	45	19	210	125	32	17.5	65
30 min.	173	35	17.5	55	113	30	16.5	32
1 hr.	178.5	20	15.5	49	118	20	14	20
2 hr.	169	13	13.5	36	115	11	13	8
3 hr.	161	12	14	12	115	7	12.5	6
4 hr.	164	7½	12	7½				

*Hoffman-La Roche. Each subject received 0.8 mg. of heparin per kilogram of body weight.

†The thrombin was prepared according to the directions of Eagle and modified by Quick.[1] The product obtained which is designated as full strength (F. S.) will clot two parts of oxalated human plasma in three seconds. When the thrombin is diluted 1:5 (1/5 S.) it clots plasma in seven seconds.

In Table II the effect of intravenously injected heparin on dogs is recorded. It will be observed that the five brands of heparin had approximately the same effect in reducing the number of circulating platelets. A dose of 0.5 mg. (55 units) per kilogram of body weight seems to be the critical amount since it sometimes produced thrombocytopenia and sometimes did not. Above this dose, a fall in platelets unfailingly was observed.

To determine how soon the platelets are affected after the injection of heparin, blood was taken every minute for five minutes. One minute after the injection all the platelets were clumped but still in the blood stream. A minute later single platelets again appeared and the clumps were diminishing. At the end of five minutes all agglutinated platelets had disappeared from the blood. (Table III.)

TABLE II. THE EFFECT OF INJECTING HEPARIN INTRAVENOUSLY IN DOGS ON THE CIRCULATING PLATELETS

BRAND OF HEPARIN	ABBOTT		ABBOTT		HOFFMANN-LA ROCHE		LEDERLE		LILLY		UPJOHN		HOFFMANN-LA ROCHE	
DOSE PER KG. OF BODY WEIGHT	2 MG.		1 MG.		1 MG.		1 MG.		1 MG.		1 MG.		0.5 MG.	
DOG	F.		S.		S.		M.		G.		M.		F.	
TIME (MIN.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)
0	391	6	397	6	425	6	262	6	219	6	341	6	352	6
5	25	33	162	20	132	23	15*	20	75*	25	111	29	172	14
15	247	27	431	11	300	16.5	197	11.5	111	19	341	21	94	10.5
30	295	21	376	8.5	391	13	274	10	154	14	354	14.5	371	9

*Moderate agglutination.

TABLE III. THE SPEED WITH WHICH PLATELET AGGLUTINATION AND THROMBOCYTOPENIA OCCUR AFTER INJECTION OF HEPARIN IN THE DOG (DOG G.; WEIGHT, 16 KG.; HEPARIN* INJECTED: 16 MG.)

TIME (MIN.)	PLATELETS (THOUSANDS)	AGGLUTINATION
0	193	None
1		Complete
2	65	Nearly complete
3	25	Extensive
4	19	Little
5	25	None

*Hoffman-La Roche.

The various brands of heparin produced no thrombocytopenia in rabbits as seen in Table IV. The platelet counts were made on arterial blood.

Only a few studies of the effect of injecting peptone in rabbits were made since they yielded results similar to the ones obtained earlier by Quick, Ota, and Baronofsky.[7] Peptone causes a marked drop in the platelet count but produces little or no heparinemia (Table V), whereas in dogs enough heparin is often poured into the blood to render it incoagulable.

From the results obtained in the present study one can conclude that the intravenous injection of heparin in doses as large as 5 mg. per kilogram of body weight in rabbits and 0.8 mg. in man causes no demonstrable diminution of the circulating platelets. In dogs, on the contrary, doses as small as 0.5 mg. per kilogram of body weight may cause a marked but transient fall in the platelet count. This is due to the agglutination of these cells, and presumably to the subsequent removal of these clumps by the capillary bed. The agglutination as shown in Table III occurs almost immediately after the heparin is injected. Promptly following this reaction, restoration of the platelet count begins to occur and may be amazingly rapid. In some instances the count may be one-third of the original level five minutes after the injection and completely normal ten minutes later.

Since heparin does not cause thrombocytopenia in either man or rabbit, it is clear that this agent per se has no influence on platelets. To imply that the thrombocytopenia following high ionizing irradiation is the resultant of heparinemia is certainly open to question. To be sure, the injection of peptone in dogs causes heparinemia, thrombocytopenia, and histamine production, but in the rabbit the same dose of peptone produces only thrombocytopenia. It is unlikely that the heparinemia in dogs is the cause of the abrupt drop of platelets. It might also be stated that injection of histamine likewise causes no change in the platelet count,[7, 8] consequently this agent can not be implicated in the thrombocytopenia observed in shock. On the basis of present evidence it seems reasonable to conclude that the three effects of shock seen in dogs are independent of each other except that their causative agent may perhaps be the same. One must not ignore the possibility, however, that the coagulation defect which we have recently demonstrated in thrombocytopenia[9] when accentuated by heparinemia may convert the vascular hyperpermeability due to histamine into a serious hemorrhagic condition. It is doubtful if heparin alone or even in the sole presence of a thrombocytopenia will cause petechiae or a prolonged bleeding time. These later effects assuredly are the result of a vascular factor.

TABLE IV. THE EFFECT OF HEPARIN GIVEN INTRAVENOUSLY ON THE PLATELET COUNT IN THE RABBIT

BRAND OF HEPARIN	ABBOTT		ABBOTT		HOFFMANN-LA ROCHE		LEDERLE		LILLY		UPJOHN	
DOSE PER KG. OF BODY WEIGHT	5 MG.		2 MG.		2 MG.		2 MG.		2 MG.		2 MG.	
TIME (MIN.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)	PLATELETS (THOUSANDS)	THROMBIN TITRATION ($\frac{1}{2}$ S.) (SEC.)
0	500	4	320	4	268	$3\frac{1}{2}$	189	4	271	4	188.5	4
5	478*	30	290	12.5	259	24	187	26	289	22	177.5	24
15	496	22	316	9.5	247	13	210	18	398	12	181	13
30	425	13	390	5.5	275	$59\frac{1}{2}$	164	10.5	270	8.5	201	9.5

*Slight agglutination.

TABLE V. THE INFLUENCE OF INTRAVENOUSLY INJECTED PEPTONE ON THE PLATELETS IN THE RABBIT (300 MG. OF PEPTONE* PER KILOGRAM OF BODY WEIGHT)

TIME (MIN.)	PLATELETS (THOUSANDS)	AGGLUTINATION	THROMBIN TITRATION ($\frac{1}{5}$ S.) (SEC.)
0	279	None	6.5
5	125	Almost complete	6.5
15	60	Extensive	6.5
30	125	Moderate	6.5

*Witte, Rostock, Germany.

It is curious that heparin causes a thrombocytopenia in dogs but not in man or rabbits. One can proffer several possible explanations. The first is that the purified heparin may still contain a trace of impurity to which the dog but not the rabbit or man is susceptible. A second hypothesis is that heparin reacts with a plasma constituent, thereby producing an agent which causes the agglutination of platelets. According to this view, dog plasma contains this factor but rabbit and human plasma do not. A third hypothesis is that dog plasma lacks protective factors that would counteract the physicochemical alteration induced by heparin. It is to be noted that the effect of heparin on the platelets is immediate, and that the platelets actually begin to increase while the heparinemia is still at its height. This shows that the presence of even a high concentration of heparin does not depress the platelet count but actually allows a rapid restoration which is brought about very likely from the liberation of the individual cells from the agglutinated clumps.

There are probably two types of platelet agglutination. The first is the type induced by heparin in dogs by peptone and perhaps by many other agents as Achard and Aynaud noted as early as 1908.[10] Perhaps this type of agglutination is brought about by as simple a change as the removal of an electric charge needed to maintain the discreteness of the platelet. The second type of agglutination is apparently the result of labilization of the platelet by thrombin. In this type the platelets become sticky, readily adhere to rough surfaces, and disintegrate, thereby liberating an enzyme that activates the plasma thromboplastinogen. The platelets remain stable whenever the production of thrombin is inhibited. This accounts for the stability of platelets in hemophilia, in marked Dicumarol hypoprothrombinemia, in oxalated and citrated plasma, and in heparinized plasma. Heparin however does not prevent the first type of agglutination as Quick, Ota, and Baronofsky[7] and Fidlar and Waters[11] have shown.

From a practical point of view the agglutination of platelets by heparin is of little moment since it does not occur in man and since even in dogs it is very transitory and unaccompanied by any untoward effects. Heparin depresses platelet agglutination of the second type by inhibiting the formation of thrombin as well as by neutralizing it. Because of this action, heparin is a reliable drug for prophylaxis against intravascular clotting.

SUMMARY

The effect of intravenously administered heparin on the circulating platelets was studied in man, rabbits, and dogs. Five brands of commercial heparin were used. No significant agglutination or thrombocytopenia was observed in man

and rabbits even after relatively large doses. In dogs a dose of 1 mg. per kilogram of body weight invariably caused agglutination of platelets and a subsequent transitory thrombocytopenia. Probable causes for this are discussed.

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EFFECT OF VITAMIN K ON DICUMAROL-INDUCED HYPOPROTHROMBINEMIA IN RATS

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MOST of the reports dealing with the effect of vitamin K on dicumarol-induced hypoprothrombinemia have been based on one or another of the one-stage methods of prothrombin estimation.[1-7] It has been shown that these methods depend not only on the concentration of prothrombin but also on the rate of conversion of prothrombin to thrombin.[8] Thus the one-stage tests lose some of their reliability as a means of measuring prothrombin concentration unless factors which may influence conversion rate are eliminated. In recent years several workers have established that another constituent present in normal plasma influences the conversion of prothrombin to thrombin.[9-13] In addition, the presence of a distinct inhibiting substance in the blood of animals treated with a hydrogenated derivative of Dicumarol has been postulated.[14, 15] This substance is considered to be similar to but not identical with heparin. Some of the clinical reports are based on the cessation of spontaneous bleeding which is admittedly influenced by many variables.

The two-stage method of prothrombin determination[16] separates the conversion phase from the clotting phase and thereby eliminates the rate of conversion of prothrombin to thrombin as a factor in prothrombin measurement. It seems pertinent, then, to examine by this method the effect of Dicumarol on the prothrombin level of blood and the modification of that effect by vitamin K.

MATERIALS AND METHODS

Adult albino rats of the Sprague-Dawley strain were used. They were maintained on a diet which contained adequate amounts of vitamin K.* Water and diet were fed ad libitum.

Suspensions of 3,3′-methylenebis (4-hydroxycoumarin) were prepared as follows: 70.2 mg. Dicumarol† were suspended in 32.1 c.c. of distilled water. To effect increased dispersion of the drug suspension, 3.0 c.c. of 0.1N sodium hydroxide were added.

Menadione‡ (2-methyl-1,4-naphthoquinone) was dissolved in corn oil (Mazola) so that 50 mg. were contained in each cubic centimeter of the solution.

Hykinone§ (menadione bisulfite) was used as supplied by the manufacturer. One cubic centimeter contains the equivalent of 2.5 mg. of menadione.

Dicumarol and menadione were given by stomach tube, and Hykinone was given by intraperitoneal injection. Both the vitamin K preparations and Dicumarol were given as daily doses.

Blood, obtained by jugular puncture, was drawn into 1.85 per cent potassium oxalate. The blood and anticoagulant were carefully mixed and placed in a hematocrit tube, and the tube was set in ice water to induce rapid cooling. After centrifugation in a refrigerated room (5° C.) prothrombin titration by the two-stage method[16] was performed on the plasma.

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*Rockland Rat Diet. (Complete.)
†E. R. Squibb & Sons, New York, N. Y.
‡Eli Lilly & Company, Indianapolis, Ind.
§Abbott Laboratories, North Chicago, Ill.

RESULTS

I. *Dicumarol Alone.*—A. In the first experiment the animals were given daily doses of constant size of Dicumarol, and the prothrombin level (expressed in per cent of normal) was followed for a period of from two weeks in some

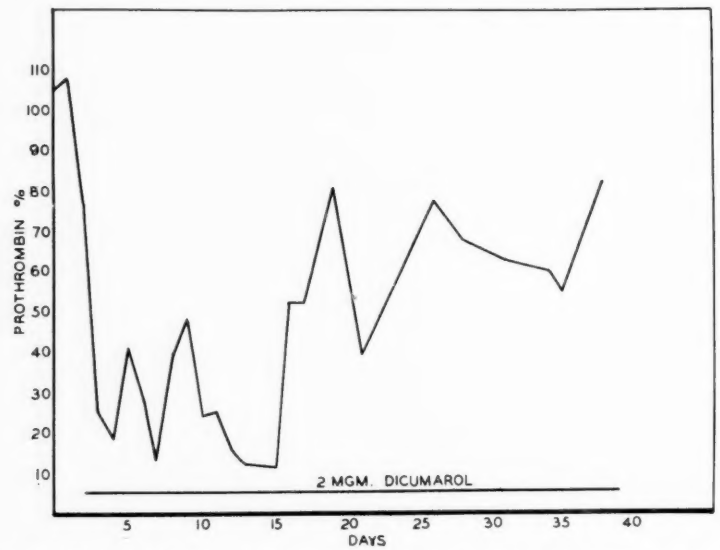

Fig. 1.—The effect of daily doses of Dicumarol on the prothrombin level of rat plasma.

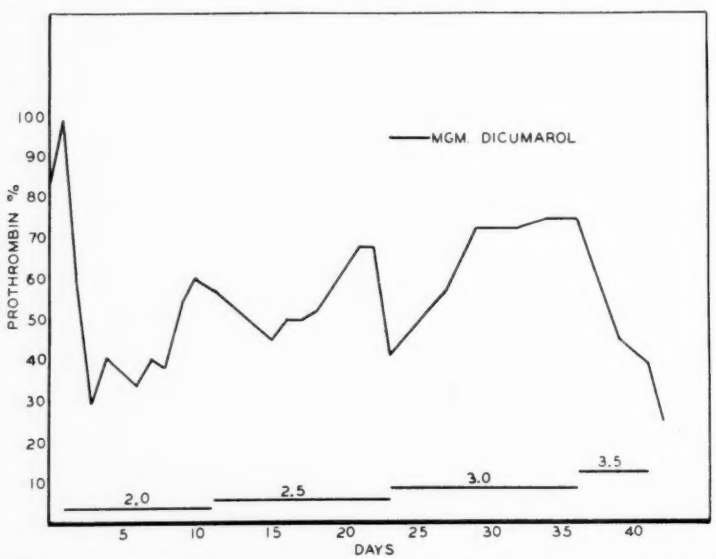

Fig. 2.—The effect of daily doses of increasing size on prothrombin level of rat plasma.

cases to as long as forty-three days in others. Fig. 1 shows a typical curve. There was a definite tendency for the animals to develop a tolerance to the drug. After approximately two weeks the prothrombin level tended to rise, and at the end of three to four weeks it commonly approached normal values. The daily variations in prothrombin level of Dicumarol-treated rats were great, as much

as 40 per cent in some instances. These variations are greater than can be explained on the basis of technical error in performing the determinations. Repeated determinations on many occasions showed that the variations in prothrombin level were genuine.

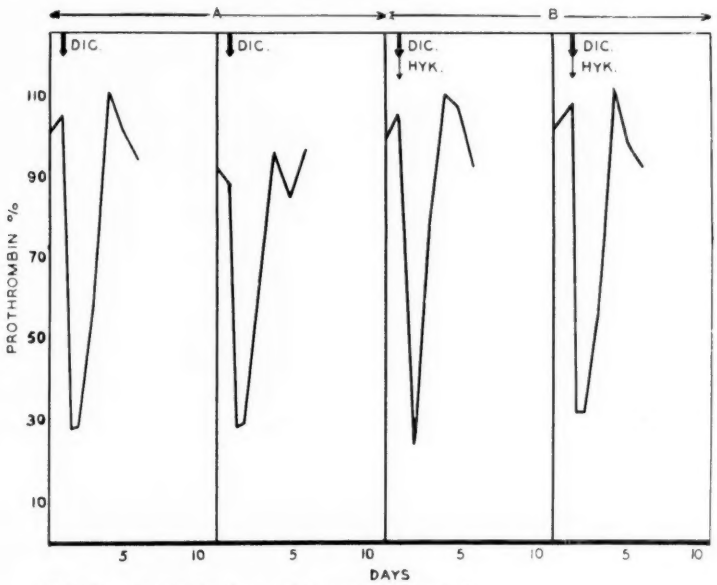

Fig. 3.—*A*, Single dose of 4 mg. Dicumarol.
B, Single dose of 4 mg. Dicumarol and 4 c.c. Hykinone.

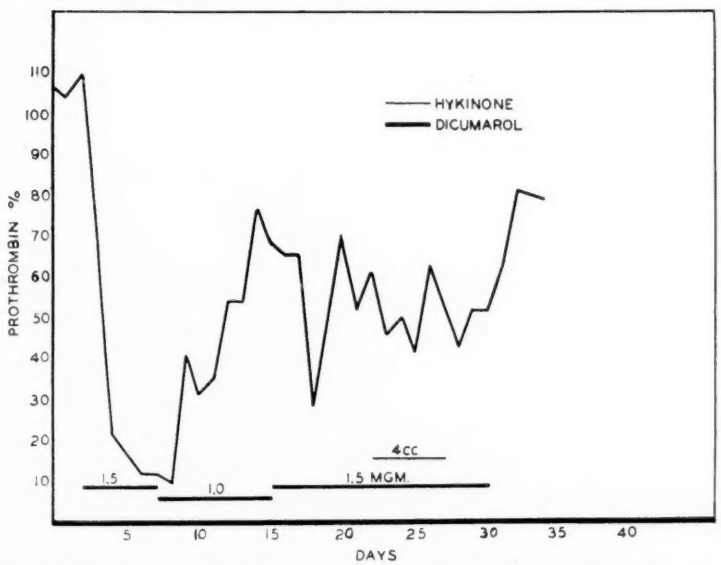

Fig. 4.—The effect of Hykinone given during administration of Dicumarol.

B. In another group of rats the dosage was gradually increased in an effort to keep the prothrombin at a low level. Virtually the same type of curve was obtained (Fig. 2) although the prothrombin level tended to remain low for a longer time. Succeeding large doses had less effect than earlier small doses.

C. In the third experiment the effect of a single dose of Dicumarol was studied (Fig. 3, *A*). A dose of 4.0 mg. of Dicumarol was given, and the prothrombin level was followed for two days after it had returned to normal. The maximum hypoprothrombinemic effect was obtained within forty-eight hours and recovery was complete within ninety-six hours after the time of medication.

In the foregoing experiments the prothrombin level promptly returned to normal when Dicumarol administration was stopped. This occurred within ninety-six hours after administration of the last dose of Dicumarol. Recovery after repeated doses was at essentially the same rate as after a single dose.

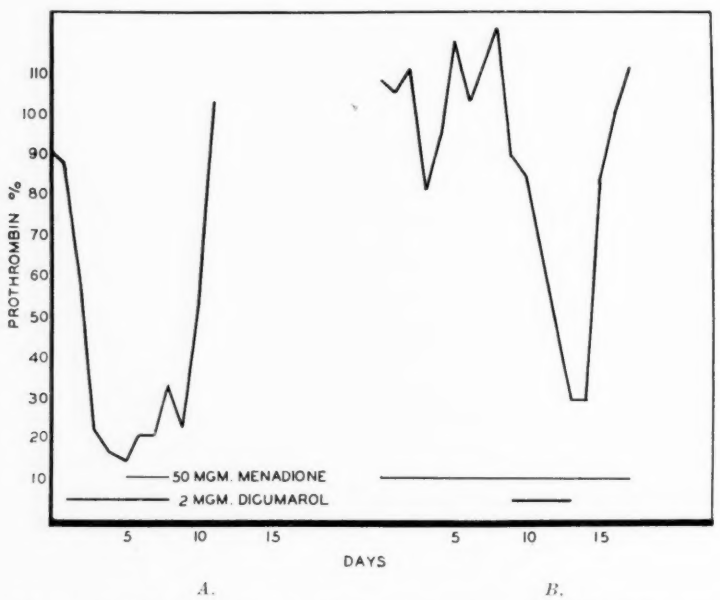

Fig. 5.—*A*, Left-hand curve. The effect of menadione given immediately preceding discontinuance of Dicumarol on the rate of recovery from the Dicumarol effect.
B, Right-hand curve, Menadione preceding, during, and after Dicumarol administration.

II. *Dicumarol and Vitamin K.*—A. Hykinone (4.0 c.c.) had no detectable effect when given to rats receiving daily doses of Dicumarol (Fig. 4). No change was observed which was greater or in a different direction than frequently was observed with continued administration of Dicumarol alone.

B. In a second group of animals large doses of menadione (50 mg. daily) were given for a period of one to two weeks before Dicumarol administration was begun, and it was continued during the period of Dicumarol administration (Fig. 5, *B*). A standard dose of 2.0 mg. of Dicumarol was given. The hypoprothrombinemic effect of the Dicumarol in these animals was similar to that in animals which had received no menadione (Fig. 1). Upon discontinuing the Dicumarol, recovery occurred at the same rate as in rats which had received no menadione.

C. A third group of animals was given Dicumarol for at least five and as long as twenty-four days. During the last three to six days of Dicumarol administration 50 mg. of menadione were also given (Fig. 5, *A*). When both

Dicumarol and menadione were stopped, the prothrombin level rose to normal at the same rate as it did when the administration of Dicumarol was stopped in animals which had received no menadione (Fig. 6). In other animals in the

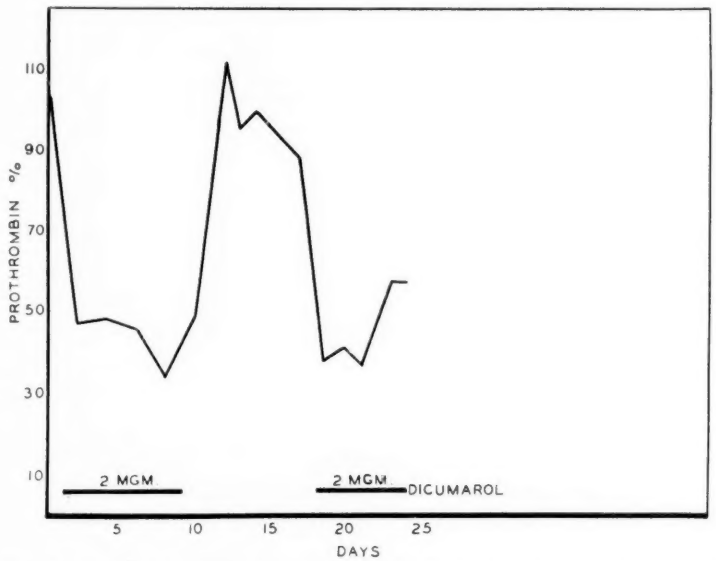

Fig. 6.—Rate of recovery from Dicumarol effect after drug was stopped.

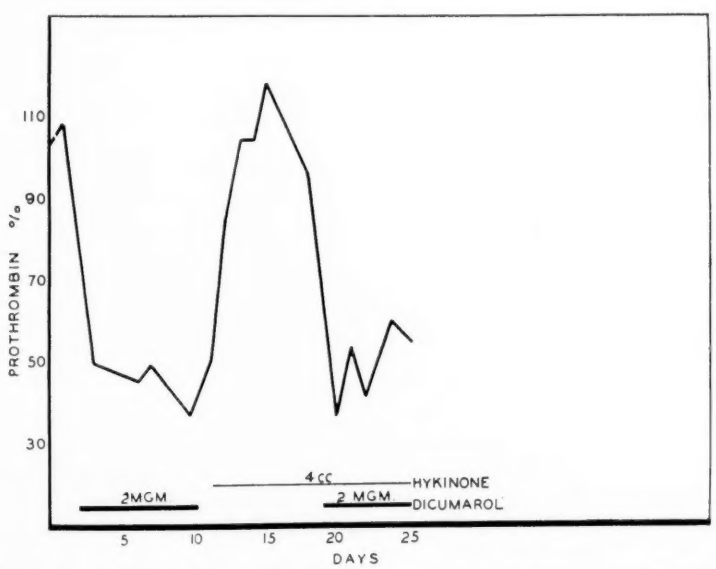

Fig. 7.—Rate of recovery from Dicumarol effect when Hykinone was given after the Dicumarol was stopped. Note that the prothrombin was reduced when Dicumarol was given again in spite of the continued administration of Hykinone.

same experiment vitamin K was given as Hykinone (dose equivalent to 10 mg. menadione) intraperitoneally. The results were the same as with menadione in corn oil by stomach tube.

D. In a fourth group of animals, Dicumarol was given for a period of five to ten days and then followed by the administration of 4.0 c.c. of Hykinone daily during the period of recovery (Fig. 7). Recovery occurred at the same rate and to the same degree as in rats which received no Hykinone (Fig. 6). When Dicumarol was administered immediately following complete recovery a response similar to that obtained with the initial dose was observed in both groups.

E. In single dose experiments the prothrombin curves of rats given Dicumarol and Hykinone together (Fig. 3, *B*) were similar to those of rats which received Dicumarol alone (Fig. 3, *A*).

DISCUSSION

There are two outstanding differences in technique between these experiments and those reported by Overman and co-workers.[2] Their animals were given a diet low in vitamin K while ours were given a diet which contained adequate amounts of it. The prothrombin determinations in their experiments were done by a one-stage method using dilute plasma while ours were done by the two-stage method. We wished to parallel as closely as possible clinical conditions in which the diet is not likely to be deficient in vitamin K, and to measure prothrombin concentration only.

The results of clinical studies of the effect of vitamin K on Dicumarol-induced hypoprothrombinemia are confused somewhat because additional measures such as discontinuance of Dicumarol administration, blood transfusions, and other agents have been used to correct the hemorrhagic tendency. The clinical studies also lack the control possible in experimental animals. In many cases vitamin K was given at a time when the prothrombin level might have been expected to be rising due to the development of tolerance to the drug. Another possible cause for the great variation in clinical results is the wide diversity of methods used to estimate prothrombin. These vary from simple observation of cessation of spontaneous bleeding to the dilute plasma technique of Link. There is some evidence[17] that the Quick and Link methods cannot be substituted for each other. This suggests that they may measure different things. They, as all the one-stage methods, are certain to be influenced by plasma factors other than prothrombin concentration. There is also evidence[18] that the dilute plasma techniques have much greater standard deviations on duplicate determination than do whole plasma techniques. Even though the sensitivity is increased by dilution of the plasma, the increased possibility for error may be another reason for inconsistencies of results.

It is possible that vitamin K administration affects factors which govern prothrombin conversion as well as those which control its concentration. This could explain the protective action of vitamin K against Dicumarol which has been reported by workers using the one-stage methods of prothrombin estimation. It would also explain why this action is not demonstrable by the two-stage method.

SUMMARY

1. The prothrombin level, as determined by the two-stage method, varied considerably from day to day in rats treated with Dicumarol. There was a

definite recovery phase soon after the initial fall in prothrombin caused by Dicumarol. Later the animals showed a decided tendency to escape from the effect of the drug.

2. Menadione and menadione bisulfite in large doses had no detectable counteracting effect on the prothrombin level of rats whether given before, during, or after Dicumarol administration.

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VENTRICULAR IRREGULARITIES INDUCED BY SYMPATHO-ADRENAL DISCHARGE AND CHLOROFORM

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CHENOWETH,[1] working with dogs, reported that the inhalation of the vapor of various lipotropic hydrocarbons including toluene, xylene, petroleum ether, and gasoline, sensitizes the mammalian ventricle to epinephrine with resultant extrasystoles, tachycardia and fibrillation, simulating the well-known chloroform-epinephrine syndrome.[2, 3, 4, 5]

In discussing his findings, Chenoweth suggests that human subjects sensitized by the inhalation of such vapors might be susceptible to ventricular fibrillation, if subjected to emotional stress which would liberate epinephrine.

The electrocardiographic studies presented here, on dogs in which a sympatho-adrenal discharge was pharmacologically induced and to which chloroform was administered during the height of such induction, seem to substantiate that suggestion.

Dogs weighing 2 to 15 kilograms were put under basal anesthesia by intraperitoneal injections of sodium pentobarbital solution carrying 37.5 mg. per kilogram of body weight. Control presurgical electrocardiographic records were then taken using the three standard leads. Tracheal and carotid cannulae were then inserted and the femoral vein was exposed, and after a thirty-minute rest period following the surgery, an electrocardiographic Lead II was recorded. Each animal was given from 0.5 to 2.0 mg. of atropine sulfate intravenously, depending on the weight of the animal, and five minutes later 1.5 mg. of physostigmine salicylate. Control records (electrocardiographic Lead II) were taken three minutes after each drug injection. Five minutes after the physostigmine was given, 0.2 to 4.0 mg. of acetylcholine bromide (depending on the weight of the animal) were injected. At the onset of the resulting blood pressure rise, the administration of chloroform vapor was begun and continued uninterruptedly until a danger point as indicated by marked fall in blood pressure and apnoea was reached. Continuous electrocardiographic Lead II, blood pressure and respiration records were obtained from the time just prior to the acetylcholine administration until the cessation of the chloroform anesthesia.

In this sequence of injections, presumably the muscarinic action of the acetylcholine would be blocked by the atropine and the destructive action of cholinesterase on the acetylcholine held in check by the physostigmine, leaving the nicotinic action of the acetylcholine unrestrained. The nicotinic effects of acetylcholine are believed to include a sympatho-adrenal discharge and the mobilization of epinephrine or an epinephrine-like substance.[6, 7, 8] If the nicotinic

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effect were great enough, it seemed that the intrinsic epinephrine liberated by the animal could affect the chloroform-sensitized ventricular complex just as intravenous injections of epinephrine.

Nine dogs of both sexes and various weights were studied. No differences in results attributable to sex or weight were found. There was however a wide variation in sensitivity as indicated by the fact that in three dogs a nicotinic effect could not be induced, in spite of different dosages of drug administration.

In the six dogs in which nicotinic responses were evoked, the following abnormal electrocardiographic findings were obtained during the reaction to chloroform.

Three dogs: Ventricular extrasystoles, varying from single extra beats to short bursts of twenty or more ventricular extrasystoles.

Two dogs: A persistent ventricular tachycardia of several minutes' duration.

One dog: A ventricular tachycardia changing to fibrillation during which the animal expired.

In all instances except the last the normal electrocardiographic pattern returned following the termination of the abnormal cardiac activity.

Aside from the changes listed, no other deviations in the electrocardiogram were noted, and these changes occurred only during chloroform inhalation following the injections of atropine, physostigmine, and acetylcholine. No such changes in electrocardiogram were noted following the injections of atropine, physostigmine, and acetylcholine into animals receiving no chloroform.

This was to be expected, for Myerson and co-workers[9] have shown that, although physostigmine and choline ester may produce severe heart block, atropine prevents this block. In the present experiments atropine and physostigmine always preceded the injection of the acetylcholine.

The findings reported here strengthen the suggestion of Chenoweth[1] that intrinsic epinephrine if liberated in sufficient quantity through emotional stress could present a serious hazard through its effect on the ventricular complex sensitized to various lipotropic hydrocarbons.

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LABORATORY METHODS

A MICROMETHOD FOR THE DETERMINATION OF THE HUMAN ALBUMIN, GLOBULIN, AND HEMOGLOBIN CONTENTS

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IN OUR previous communications[1, 2] it was reported that the estimation of human albumin in serum or other body fluids by means of the precipitin reaction gives an accuracy which approximates that of electrophoretic analysis, the generally accepted standard, and requires only a minute amount of the protein. This latter situation makes it possible for the development of a micromethod for the determination of albumin, total protein, and hemoglobin in capillary blood taken by finger puncture. These determinations were performed with 0.2 ml. of blood which was then diluted with a known volume of 0.85 per cent saline solution, containing enough heparin to prevent coagulation. The red cells were then removed by centrifugation and laked with a dilute ammonia solution for hemoglobin determination with a photoelectric colorimeter. Aliquot samples of the supernatant were used for determination of total protein nitrogen and albumin nitrogen. The former was estimated by measuring the turbidity of the trichloroacetic acid precipitate; the latter, by the precipitin method. The difference between the total protein nitrogen and albumin nitrogen was taken as the total globulin nitrogen.

Reagents.—

1. A dilute ammonia solution. Approximately 5 ml. of concentrated ammonia solution (28 per cent ammonia by weight) are diluted with water and made to 1 liter.

2. A 7.5 per cent trichloroacetic acid solution. Seventy-five grams of Merck's reagent grade trichloroacetic acid are dissolved with water and made to 1 liter.

3. A normal saline solution; 17.0 grams of chemically pure NaCl are dissolved with water and then made to a volume of 2 liters. To one liter of this solution are added 10 mg. of heparin.

4. Antihuman albumin rabbit serum. Sera of rabbits immunized against human albumin can be prepared according to our previously described procedure.[1] The pooled sera is kept in a frozen state until shortly before use, and filtered if necessary, after thawing. It is then diluted exactly with an equal volume of the saline solution.

Procedure.—

Blood is drawn from the tip of the finger by puncturing with a dry lancet until it flows freely. A 0.20 ml. sample is measured with a capillary pipette and transferred quantitatively into a 15 ml. centrifuge tube containing 10.0 ml. of 0.85 per cent saline solution with heparin. The suspension is then centrifuged for fifteen minutes, and the supernatant (referred to as SU) is carefully removed by a capillary tube and saved for the determination of the albumin nitrogen, and the total plasma protein nitrogen.

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Determination of Hemoglobin.—

The red cells at the bottom of the tube are hemolyzed with 5 ml. of the dilute ammonia solution. After laking, 2 ml. of the hemoglobin solution are then pipetted into a Klett-Summerson tube containing 4 ml. of the same ammonia water. Thus, the final sample contains an equivalent of 0.0133 ml. of blood. The amount of hemoglobin is then determined colorimetrically, using a green filter of 540 millimicrons. To express the results in grams per cent (see columns A and B in Table I), it is necessary to determine the oxyhemoglobin factor* by correlating the colorimeter readings with oxygen combining capacity.

Determination of Total Protein Nitrogen.—

Duplicate samples of 3.0 ml. of the supernatant (SU) are pipetted into two Klett-Summerson tubes to each of which are then added 2.0 ml. of 7.5 per cent trichloroacetic acid solution from a 10 ml. burette. The turbidity produced is then measured in a Klett-Summerson

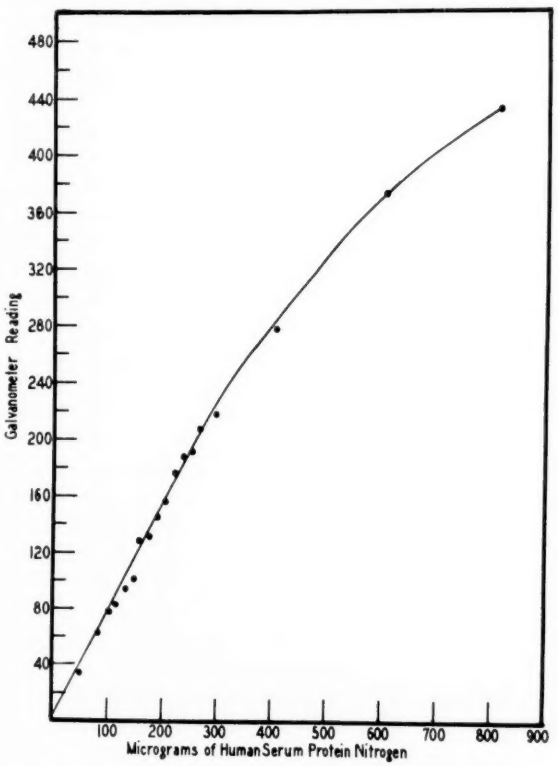

Fig. 1.—A standard curve showing the relationship between turbidity readings and micrograms of serum protein nitrogen.

tube after standing at least five minutes. The readings remain constant for more than two hours after precipitation. The amount of protein in the precipitates can then be estimated from a previously standardized curve, which is obtained by measuring the turbidity of the trichloroacetic acid precipitate of a known amount of serum protein nitrogen. The results of a typical experiment are plotted in Fig. 1. To substantiate that such a relationship is applicable to normal as well as abnormal serum, we have compared the Kjeldahl nitrogen with the turbidity nitrogen.

**Determination of factor.* Procure sample of fresh blood and determine oxyhemoglobin content by either the oxygen capacity or the carbon monoxide capacity method. Run a colorimetric determination on duplicate or triplicate 0.02 ml. portions of this blood. From the known hemoglobin content of the blood, the factor may be determined as follows:

$$\frac{\text{Gm. per cent hemoglobin}}{\text{Colorimeter reading}} = \text{Oxyhemoglobin factor}$$

TABLE I. RESULTS OF ANALYSES OF THE HEMOGLOBIN, ALBUMIN, AND GLOBULIN CONTENTS OF THIRTEEN INDIVIDUALS

NAME	HEMOGLOBIN		TOTAL PLASMA PROTEIN IN MG. N/ML.			ALBUMIN NITROGEN			% ALBUMIN NITROGEN IN:		% GLOBULIN DIFFERENCE (K)
	GALVANOMETER READINGS (A)	GRAM % (B)	KJELDAHL METHOD (C)	TURBIDITY (D)	% DEVIATION KJELDAHL METHOD (E)	TURBIDITY READING (F)	ALBUMIN N/SAMP. (G)	ALBUMIN N/ML. BLOOD (H)	KJELDAHL NITROGEN (I)	TURBIDITY NITROGEN (J)	
I. H.	436	16.2	6.80	6.53	−4.0	107 – 106	0.0290	2.96	44	45	55
B. F. C.	535	19.1	7.58	7.89	+4.1	106 – 104	0.0280	2.86	38	36	64
I. E.	410	15.2	8.33	7.85	−5.8	103 – 103	0.0270	2.75	33	35	65
I. H.	490	18.2	7.65	7.38	−3.5	109 – 106	0.0295	3.01	39	41	59
C. F.	458	17.0	6.87	6.83	+0.6	104 – 103	0.0275	2.81	41	41	59
J. C.	475	17.6	7.31	7.38	+0.1	88 – 90	0.0210	2.14	29	29	71
I. G.	428	16.0	7.96	7.79	−2.1	93 – 95	0.0230	2.35	29	30	70
I. P.	465	17.2	7.17	7.02	−2.1	106 – 104	0.0280	2.86	40	41	59
S. D.	500	18.5	7.14	7.24	+1.4	98 – 102	0.0260	2.65	37	37	63
L. B.	455	16.9	7.82	7.38	−5.6	107 – 106	0.0290	2.96	38	40	60
F. N.	455	16.9	7.65	7.58	−0.1	97 – 96	0.0245	2.50	33	33	67
C. A.	520	18.9	6.80	6.72	−0.1	102 – 99	0.0265	2.70	40	40	60
R. M.	506	17.1	6.70	7.00	+4.5	101 – 100	0.0260	2.65	40	38	62
		Ave. 17.3		Ave. Dev. 2.6 Stand. Dev. 2.01					Ave. 37	Ave. 37	

The data (see Table II) are separated into four groups according to the percentage of albumin in the serum, ranging from 31 to 70 per cent. The amount of total serum nitrogen in each sample as determined by the Kjeldahl method is arbitrarily taken as 100 per cent with which the turbidity nitrogen is compared. The results demonstrate that the turbidity method gives a slightly higher result of less than 10 per cent, and a standard deviation of about 5. Such an agreement between these two methods may be considered as satisfactory for clinical use. The results of a typical experiment with variations of the individual samples are recorded in columns C, D, and E in Table I.

Determination of Albumin Nitrogen.—

The amount of human albumin nitrogen in the supernatant (SU) is determined immunologically according to the procedure previously described. The following modifications are introduced in order to adapt this method to the small size of sample obtained by finger puncture. Two milliliters of the supernatant (SU) are diluted with an equal volume of 0.85 per cent NaCl solution. Two 1 ml. samples of this solution, now equivalent to 0.0098 ml. of the original blood, are then added to two Klett-Summerson tubes for duplicate determinations. Four milliliters of the diluted antihuman albumin rabbit serum are added to each tube from a 50 ml. burette, and the turbidity of the immune precipitate is measured after standing at room temperature for at least thirty minutes with a Klett-Summerson photoelectric colorimeter using a blue filter of a wave length 420.

TABLE II. DETERMINATION OF THE TOTAL PLASMA PROTEIN NITROGEN BY PRECIPITATION WITH TRICHLOROACETIC ACID

PER CENT OF ALBUMIN IN PLASMA	NUMBER OF SAMPLES	TOTAL PLASMA PROTEIN NITROGEN BY TURBIDITY METHOD* % ± S.D.
31-40	6	103 ± 6.0
41-50	23	107 ± 4.0
51-60	17	106 ± 5.0
61-70	5	107 ± 5.1

S.D., Standard deviation.

*Expressed as per cent of that found by the Kjeldahl method which is taken as 100 per cent.

The amount of human albumin corresponding to the turbidity readings can be estimated from the standard curve. Multiplying the albumin content per sample (column G in Table I) by the serum dilution (i.e., 51 × 2 equals 102) gives the total albumin nitrogen per milliliter of blood (column H). The ratio of total albumin nitrogen to the total nitrogen, as determined either with the micro-Kjeldahl method (column I) or from the turbidity of the trichloroacetic acid precipitate (column J), multiplied by 100 gives the per cent of the total nitrogen as albumin. The difference between 100 per cent and the per cent of albumin nitrogen gives the per cent of globulins (column K).

Results.—

The results of a typical experiment with samples of blood drawn from thirteen individuals are summarized in Table I, in order to show the normal variations of routine determinations. They demonstrate that the agreement in total plasma protein nitrogen between the two methods (micro-Kjeldahl and turbidity) is satisfactory for clinical use. The average percentage deviation of the turbidity nitrogen from the Kjeldahl nitrogen is 2.6 per cent. The same magnitude of deviation is reflected in the calculation of per cent of albumin based on total protein nitrogen measured with either one of the two methods. The average percentage albumin in the plasma of this group of individuals is only 37 per cent.

In Table III are summarized our data from thirty-eight normal individuals. Similar results obtained by other leading methods are included for comparison. It can be noted that the average concentration of both hemoglobin and of plasma proteins in grams per 100 ml. of blood of our normal individuals is higher than that reported in the literature. Whether this difference can be attributed to the difference in the method of determination, or to the individual variation, is hard to decide on the basis of our limited number of experiments. Our average figures in the per cent of albumin in plasma approximate closely the reported figures of electrophoretic albumin,[3] but are significantly lower than those of methanol soluble albumin of Pillemer.

TABLE III. SUMMARY OF DATA FROM NORMAL MEN ON HEMOGLOBIN, PLASMA PROTEINS, AND PER CENT ALBUMIN OBTAINED BY DIFFERENT METHODS

SUBSTANCE DETERMINED	METHODS USED	
	AS PRESENTED IN THIS PAPER	OTHER METHODS
Gm. hemoglobin in 100 ml. blood	16.0-21.5	(a) 14.1-17.2[1] (b) 12.9-15.3[1]
Gm. plasma proteins in 100 ml. blood	4.46[4] 4.68[5]	3.45-4.24
Per cent albumin in plasma	44.6[4] 42.3[5]	47[2] 52[3]

1. Copper sulfate method for measuring specific gravities of whole blood and plasma. R. A. Phillips, D. D. van Slyke, V. P. Dole, K. Emerson, Jr., P. B. Hamilton, and R. M. Archibald, Published by Josiah Macy, Jr., Foundation, 1945, page 49.

2. The electrophoretic method for the analysis of albumin in human plasma by electrophoresis. Louis Pillemer and M. C. Hutchinson, J. Biol. Chem. 58, 299, 1945.

3. The methanol method for the determination of human albumin in plasma.

4. The immunologic method for the estimation of human albumin where the total nitrogen is determined by the Kjeldahl method.

5. The immunologic method for the estimation of human albumin where the total nitrogen is determined by the turbidity method.

Comparison of Albumin Content in Venous and Capillary Blood.—

Although capillary blood may, under normal conditions, be used for the estimation of blood proteins, including hemoglobin and plasma, it may not serve as a representative sample of circulating blood in conditions of severe shock or in gross generalized edema. The former situation may cause the capillary to contain 30 to 40 per cent more cells and hemoglobin than the venous or arterial blood, and in the latter situation the edema fluid from subcutaneous tissue would exude with the blood from a puncture. It is believed that proper technique of finger or ear lobe puncture will minimize such errors.

In Table IV are collected the results on the comparison of per cent of albumin in plasma of capillary or venous blood of forty-three patients with various diseases, chiefly with malignant growth. In the tabulation of results it is arbitrarily assumed that the per cent of albumin in the plasma of the venous blood is 100, with which the albumin in the plasma of capillary blood is compared. The data are segregated into three groups of ascending per cent of albumin. The results demonstrate that the per cent albumin in the plasma of the capillary blood and the per cent in the plasma of venous blood agree with each other within the accuracy of the determination.

TABLE IV. COMPARISON OF PER CENT OF ALBUMIN IN THE PLASMA OF CAPILLARY AND VENOUS BLOODS*

PER CENT ALBUMIN	NUMBER OF SAMPLES	AVERAGE PER CENT ALBUMIN IN CAPILLARY BLOOD
30-35	3	94
36-45	26	96
46-55	14	102

*The per cent of albumin in the venous blood is taken as 100, with which the per cent of albumin in the capillary blood is compared.

DISCUSSION

The pathologic conditions which can affect the plasma protein concentration and different types of anemia and polycythemia which can affect blood hemoglobin concentrations have been most adequately summarized by Kagan[4] and Phillips and associates.[5] Hence, these will not be discussed in this report. It is generally agreed that so far as the determination of plasma protein concentration is concerned, an abnormal value is a definite proof that one of the physiologic conditions controlling the concentration has been disturbed. However, a normal concentration cannot be regarded as an absence of any disturbed factor, since determination of plasma protein concentration does not take into account a hemoconcentration or dilution.

It has been demonstrated that in animals,[6, 7] as well as in man,[8] an outstanding change in plasma protein as a result of protein depletion due to either inadequate vitamin,[9] protein, or caloric intake or owing to a variety of diseases is a decrease in the albumin content, even though the total plasma protein concentration may appear normal. It is therefore believed[8, 10] that the determination of the per cent albumin in plasma is of greater clinical significance than any change of plasma concentration. A procedure such as that we have just described should be useful not only for diagnostic purposes but also for field studies or public health work, since it permits a rapid and accurate determination with a relatively small amount of sample.

SUMMARY

A micromethod is described for the determination of hemoglobin, total plasma proteins, and albumin with 0.2 ml. of capillary blood drawn by finger puncture. Blood is diluted with 0.85 per cent NaCl solution containing heparin. The suspension is centrifuged to remove red cells, which are hemolyzed for the determination of its hemoglobin content by means of photoelectric colorimeter. The total protein nitrogen in the supernatant is estimated by measuring the turbidity of the trichloroacetic acid precipitate. The albumin nitrogen in the supernatant is determined immunologically. The difference between the total nitrogen and albumin nitrogen is taken as globulin nitrogen.

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COLD HEMAGGLUTININ TEST BY A SLIDE METHOD

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THE cold hemagglutinin test recently has been widely used in the routine clinical study, particularly in the diagnosis of primary atypical pneumonia. As far as we know, all previous workers have used the test tube method employed first by Landsteiner forty-five years ago.[1] Recently Young showed that the sensitivity of the test could be increased by observing the agglutination under the microscope by transferring a drop of cell suspensions to a plain glass slide.[2] It occurred to us that the test might be performed on a hollow-ground slide on which a direct microscopic examination could then be made. The results of the comparative study of the slide and the tube methods are herewith presented.

The preparation of the cell suspensions and the sera was carried out by the usual procedures described elsewhere.[2, 3] A preliminary study on the relative sensitivity of group O erythrocytes from five donors including one of us (Hou) was made first. The results of the cold hemagglutinin test with eight sera from eight patients tested with cells of these donors were found approximately the same. Thereafter Hou's cells were used in nearly all of the tests reported in this paper.

THE SLIDE METHOD

Special medicinal droppers (one drop approximately equivalent to 0.05 c.c.) and special microslides of 10 by 4 by 0.5 cm. were used. On the surface of each slide were ten smoothly and evenly ground chambers in two rows measuring 1.5 cm. in diameter and 0.2 cm. in depth. In each of the ten chambers 1 drop of physiologic saline was placed, and the same amount of the serum to be tested was then added to the first chamber. After the saline and the serum had been thoroughly mixed in the first chamber, 1 drop of the mixture was transferred to the second chamber. Similarly, twofold serial dilutions of the serum from 1:2 to 1:512 were made. No serum was added to the tenth chamber which served as a saline control. Finally 1 drop of 1 per cent cell suspensions was added to each of the ten chambers. The slide, after being shaken, was placed in the refrigerator at 0 to 4° C for two hours. At the end of this period the slide was taken out from the refrigerator, shaken gently, and examined immediately under the microscope with a low-power objective. The cell suspension showing barely visible agglutination was taken as the end point. Titers were recorded in terms of the final dilution of the serum multiplied by two. The specificity of the test was checked by the disappearance of the agglutination after the slide remained in an incubator at 37° C. for one hour.

Aside from the regular slide method, tests in which dilutions were first made in test tubes with pipettes and later performed on the slide were made simultaneously on sixty-two specimens to determine the accuracy of the dilution by the dropper herein described. The following results were obtained: Thirty-nine out of a total of sixty-two specimens yielded the same titers by both methods of dilution. Except for one, the specimens showed a difference in titers of not more than twofold dilution; of these, thirteen gave higher titers in tests with pipette dilution and nine in those with dropper dilution. The last one showed a higher titer in the test made with the dropper dilution (fourfold). It seems reasonable to conclude that the method of dilution with the dropper is accurate enough for practical use.

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Among the workers on cold hemagglutinin test there is a general agreement that it is best to examine the serum-cell mixture by the tube method after refrigeration for fourteen to eighteen hours.[2] Therefore during the present study many tubes and slides were examined for agglutination after one, two, three, and eighteen hours of refrigeration at 0 to 4° C. Cold hemagglutinin titers nearly always reached the maximum at two hours for the slide method and eighteen hours for the tube method.

RESULTS

During the period between May, 1946, and April, 1948, 509 blood specimens were collected from sixty-two normal individuals and 302 patients. Each of these specimens was tested according to the slide technique described. The tube test was simultaneously performed by Rose's method.[3] The results obtained are summarized in Tables I and II.

TABLE I. CORRELATION OF COLD HEMAGGLUTININ TITERS BY SLIDE AND TUBE METHOD

SLIDE / TUBE	NEG.	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	TOTAL
Neg.	26	33	65	48	20	1	0	0	0	0	193
1:4	1	2	17	33	17	3	0	0	0	0	73
1:8	2	1	22	31	29	9	0	0	0	0	94
1:16	0	1	1	11	22	15	1	0	0	0	51
1:32	0	1	0	5	15	15	7	1	0	0	44
1:64	0	0	0	0	2	6	17	3	0	0	28
1:128	0	0	0	0	1	0	6	5	3	0	15
1:256	0	0	0	0	0	1	1	4	1	1	8
1:512	0	0	0	0	0	0	1	0	1	0	2
1:1024	0	0	0	0	0	0	0	0	0	1	1
Total	29	38	105	128	106	50	33	13	5	2	509

Table I shows the correlation of the cold hemagglutinin titers between the slide and the tube methods. It is obvious that the slide method is more sensitive than the tube method. There were only ninety-four (18.5 per cent) specimens whose cold hemagglutinin titers were the same by both methods, and eighteen (3.5 per cent) specimens whose cold hemagglutinin titers by slide method were

TABLE II. DISTRIBUTION OF MAXIMUM TITERS OF COLD HEMAGGLUTININS BY SLIDE METHOD IN 364 SUBJECTS

CLINICAL GROUP	COLD HEMAGGLUTININ TITERS										
	NEG	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	TOTAL
Normal individual	1	6	16	21	14	4	0	0	0	0	62
Primary atypical pneumonia	0	0	0	0	0	1	5	4	3	2	15
Lobar pneumonia	1	3	4	4	11	5	0	0	0	0	28
Pulmonary tuberculosis	2	2	6	13	11	0	2	0	0	0	36
Lung abscess	0	1	3	2	2	1	0	0	0	0	9
Other respiratory diseases	0	3	6	3	6	4	0	1	0	0	23
Tbc. pleurisy and peritonitis	0	1	3	8	3	3	1	0	0	0	19
Kala-azar	11	3	6	23	19	8	9	1	1	0	81
Typhus fever	0	0	3	2	1	3	0	0	0	0	9
Typhoid fever	0	0	1	0	3	1	0	0	0	0	5
Acute epidemic encephalitis	1	1	2	6	2	2	1	0	0	0	15
Acute epidemic hepatitis	1	3	0	0	1	0	0	0	0	0	5
Cirrhosis of liver	2	2	4	5	11	2	7	1	0	0	34
Anemia	0	0	4	2	3	3	1	0	0	0	13
Bright's disease	0	0	1	3	1	0	0	0	0	0	5
Cancer	0	0	2	2	1	0	0	0	0	0	5

lower than those by the tube method. For the remaining 397 (78 per cent) specimens the slide method gave higher titers: twofold dilution, 141 (27.7 per cent); fourfold dilution, 156 (30.6 per cent); eightfold dilution, 76 (15 per cent); and sixteenfold dilution, 24 (4.7 per cent).

From Table II it seems clear that high titers were rare except in primary atypical pneumonia, while low titers were common in various diseases as well as in normal controls. The highest titer of cold hemagglutinins obtained in our sixty-two normal individuals was 1:64. Therefore in the present study only titers above 1:64 were considered significant.

DISCUSSION

From the foregoing study the slide method is shown to be more sensitive than the tube method. It is to be noted that the ratio of the cell and serum used in the present method is practically identical with that of the tube method. The difference in the sensitivity of the two methods as shown by the results presented in Table III can be explained only by the difference in the method of examining agglutination. The exact mechanism of this phenomenon is not yet clear.

Despite the sensitiveness of the slide method, high titers of cold hemagglutinins were still uncommon except in primary atypical pneumonia (93.3 per cent of fifteen cases). But low titers occurred more frequently in various pathologic and normal sera. It is of some interest that 23.5 per cent of thirty-four cases of cirrhosis of the liver and 13.6 per cent of eighty-one cases of kala-azar showed significantly high titers of cold hemagglutinins.

TABLE III. COMPARISON OF SLIDE AND TUBE COLD HEMAGGLUTININ TITERS BY DIFFERENT METHODS OF EXAMINATION

SERA	SLIDE TITERS	TUBE TITERS			
		2 HR.		18 HR.	
		GROSSLY	MICROSCOPICALLY	GROSSLY	MICROSCOPICALLY
1	1:32	0	1:4	1:4	1:16
2	1:32	0	1:4	1:4	1:8
3	1:16	0	1:4	1:4	1:16
4	1:16	0	0	0	1:4
5	1:32	0	0	0	0
6	1:64	0	1:4	0	1:4
7	1:16	0	1:4	0	1:4
8	1:16	0	1:4	1:4	1:8
9	1:32	0	0	0	1:4
10	0	0	0	0	0

SUMMARY

A slide method for the determination of cold hemagglutinin titer has been described which is not only more rapid but also simpler than the tube method. It does not require tubes and serologic pipettes. In a very small hospital laboratory it might be advantageous to escape the necessity of washing tubes and to be able to start another series of tests after merely rinsing off the slide.

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APPARATUS FOR PRODUCING QUANTITATIVE ISCHEMIA IN THE LIMBS OF ANIMALS

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A CONTROLLED experimental procedure for producing ischemia in the limb of an animal is difficult to attain because of physical limitations in the methods used. For instance, when a blood pressure cuff around a limb is inflated to points above diastolic pressure but below systolic pressure, various degrees of ischemia may be achieved but there is no means of quantitation. Progressive ligation of the arterial supply likewise fails to produce quantitative ischemia because of increasing collateral blood flow. This procedure, also, is not applicable to short-term experiments.

To obviate some of the difficulties mentioned, a method has been devised which allows the blood to flow unimpeded for a predetermined period and then to cease for a predetermined period. This cycle is controlled electronically, and it continues to repeat itself indefinitely. Total cessation of blood flow may be varied from one-half second to three minutes, and the period of unimpeded blood flow may be varied independently between the same limits. Limits different from these may be incorporated in the apparatus by slight electrical changes, but one-half second to three minutes has been found to be very convenient for most purposes. Accuracy of calibration is approximately 5 per cent.

The theory of the method is rapid inflation of a blood pressure cuff to an arterial cutoff pressure and, at the end of a given period, instantaneous deflation of this cuff to zero pressure.

The apparatus shown in Fig. 1 consists of a simple electronic unit and a special magnetic solenoid valve which controls the inflation and deflation of the blood pressure cuff. The electronic circuit is similar to that of the usual photographic timer, and this will not be discussed in detail because anyone familiar with electronics will understand the diagram. *R2* controls the time that electrical current flows through the solenoid valve, and *R3* controls the time that this current flow is zero. *R1* is located beneath the chassis and is for standardization purposes so that the two scales, *R2* and *R3*, may be made to equal each other. Once this resistance is set, it is not changed unless restandardization is necessary.

When current flows through the coil of the solenoid valve, the steel plunger lifts and opens the inlet valve while closing the escape valve so that air may flow from the compressor into the blood pressure cuff. When the current cuts off, the plunger falls by its own weight to the bottom of the chamber, closing the inlet valve while opening the escape valve. This allows air to flow unimpeded from the cuff through the escape valve. The tank in series with the compressor increases the rapidity of air flow into the cuff as soon as the inlet valve opens. The

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reducing valve controls the pressure in the tank and therefore in the cuff during its inflated phase; this is set at around 5 pounds pressure which is above arterial cutoff but is not sufficient to cause local pressure pain.

Transition from an inflated cuff to a totally deflated one and vice versa occurs in approximately one-fifth second. This results in very little pooling of blood in the limb, which occurs markedly when the cuff is inflated to any point below systolic pressure for a long period of time.

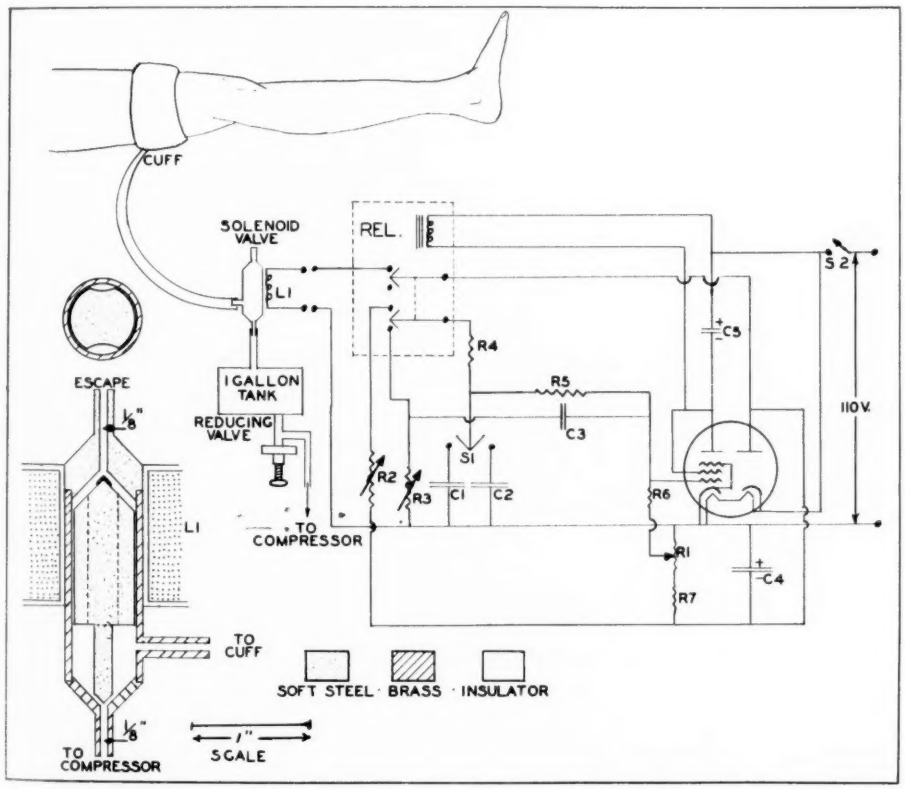

Fig. 1.—Electrical diagram and mechanical drawing of the apparatus for producing quantitative ischemia.

L1, Solenoid valve coil (3,400 turns, #34 wire)
REL., Double pole, double throw plate relay; resistance, 2,500 ohms.
S1, Single pole, double throw toggle switch
S2, Single pole, single throw toggle switch
R1, 20,000 ohm, wirewound potentiometer
R2 and *R3*, 9 megohm, carbon potentiometers, log taper
R4, 50,000 ohm, ½ watt resistor
R5, 40 megohm, ½ watt resistor
R6, 20 megohm, ½ watt resistor
R7, 100,000 ohm, 1 watt resistor
C1, 0.1 mfd., 200 volt paper condenser
C2, 10 mfd., 200 volt paper condenser
C3, 0.00025 mfd., 200 volt paper condenser
C4 and *C5*, 8 mfd., 175 volt, electrolytic condensers
Tube, 117L7/M7-GT

Using this apparatus, one can state in percentage the amount of time that the blood flows, and various states of ischemia can be reproduced. During the phase of blood flow there is a compensatory vasodilatation which appears to be four to six times normal. For this reason significant ischemia does not result until the period of zero blood flow is approximately 80 per cent of the cycle. When this is increased to 95 per cent, severe pain ensues both in man and in

dogs. Dogs in the second stage of Nembutal anesthesia will unconsciously wail loudly when this phase of ischemia is attained and will cease wailing as soon as the ischemia is lessened. Such results may be repeated within narrow limits. The reactions of animals to ischemia will be further analyzed in experiments designed to elucidate the cause of pain in Buerger's disease, Raynaud's disease, immersion foot, and acute poliomyelitis.

Erratum

In the article by LeRoy and Nalefski, "Dicumarol in Experimental Myocardial Infarction," in the August, 1948, issue of the JOURNAL, Table I should read as follows:

PROTHROMBIN TIME (SEC.)	PER CENT PROTHROMBIN TIME
6	100%
7.5	80%
10	60%
12	50%
15	40%
20	30%
30	20%

PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-First Annual Meeting

Chicago, Ill., Oct. 29 and 30, 1948

PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 29, 1948

FRIDAY MORNING, 9:15 A.M.

1. A STUDY OF PULMONARY HYPERTENSION IN CHRONIC HYPERTROPHIC PULMONARY EMPHYSEMA

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(INTRODUCED BY CECIL JAMES WATSON, M.D.)

For many years the presence of pulmonary hypertension in chronic hypertrophic pulmonary emphysema has been suspected because of the occurrence of right heart failure, the presence of right ventricular hypertrophy at autopsy, and electrocardiographic changes interpreted as indicative of right heart strain. Recently, the development of venous catheterization and the use of high-frequency manometers have permitted the accurate measurement of pulmonary arterial pressure in patients with this disease.

In fifteen patients with chronic hypertrophic pulmonary emphysema, the average pressure in the pulmonary artery was 33.9 ± 10.4 mm. Hg systolic and 20.9 ± 4.7 mm. Hg diastolic. This is compared with an average pressure of 20.1 ± 3.7 mm. Hg systolic and 8.8 ± 1.5 mm. Hg diastolic in twelve normal subjects. The mean cardiac index in the patients with emphysema was 3.38 ± 0.67 liters per minute and 3.59 ± 0.72 liters per minute in the normal subjects. Inasmuch as there is no reason to believe that the left auricular pressure is elevated in emphysema, this study indicates that the resistance in the pulmonary vascular bed is increased. The abnormally high intrapleural pressure which has been described in emphysema would account for a small portion of the rise in pulmonary arterial pressure.

A striking feature of the pulmonary arterial pressure in emphysema is the respiratory variation characterized by a marked decrease in pressure at the beginning of inspiration.

The severity of the emphysema was evaluated by measurements of vital capacity, ratio of residual air to total lung volume, pulmonary emptying rate, and the degree of oxygen unsaturation of arterial blood. There was no definite correlation between the degree of pulmonary hypertension and the severity of the emphysema as estimated by these measurements. The development of pulmonary hypertension does not appear to be completely dependent upon those factors which result in the loss of pulmonary function.

Four of the fifteen patients had definite evidence of right heart failure manifested by peripheral edema, hepatomegaly, and a markedly elevated venous pressure. In these four patients, the degree of pulmonary hypertension was not significantly different from those without right heart failure. The development of cor pulmonale and right heart failure in pulmonary emphysema does not appear to be a consequence solely of the pulmonary hypertension but probably involves other factors such as chronic anoxia.

2. STUDIES ON CARDIORESPIRATORY FUNCTION WITH THE OXYHEMOGRAPH

BEN E. GOODRICH, M.D., VIVIAN G. BEHRMANN, PH.D. (BY INVITATION), AND FRANK W. HARTMAN, M.D. (BY INVITATION), DETROIT, MICH.

Preliminary studies with the oxyhemograph have been conducted on patients subject to varying degrees of dyspnea resulting from cardiac or respiratory disabilities. Young, apparently healthy adults also were tested.

Continuous recording of the oxygen saturation of circulating hemoglobin was afforded through the use of the oxyhemograph described by Hartman, Behrmann, and Chapman. The initial arterial oxygen saturation at rest was calculated from calibration after the patient produced a stabilized level while breathing 100 per cent oxygen. After standardization, the subjects were tested in atmospheric oxygen by simple exercises of breath holding and knee bending while supine and erect. The resultant tracings were charted for comparison using arbitrary time intervals and percentage levels.

Results of these tests reveal differences in the degree and duration of changes in arterial oxygenation in various individuals. Healthy adults, asymptomatic in daily life, varied greatly in the extent of lessened oxygenation possible by voluntary breath holding. Well adults showed moderate variation in the per cent increase in the blood oxygen saturation concomitant with exercise, although cessation of exercise uniformly resulted in an immediate depression of oxyhemoglobin to or below the atmospheric level. The return to normal was prompt. The exercise was not of a degree that exhausted the subject. In patients under similar tests exercise was not associated with increased oxyhemoglobin. Patients often exhibited marked hypoxemia with a delayed recovery. Not infrequently recovery was stairstep-like in appearance.

These findings indicate that both the degree of arterial unsaturation and the character of the recovery curve may be of clinical significance. The post-exercise curve may vary as to whether recovery is spontaneous or aided by the administration of 100 per cent oxygen. Charts present typical oxyhemograms. Bronchospastic dyspnea is contrasted to structural respiratory disability. Patients with decreased cardiac reserve show abnormal curves. In these patients abnormal curves may be recorded even though symptoms are denied.

These preliminary studies indicate that the oxyhemograph together with simple tests may prove useful in the differential clinical evaluation of cardiorespiratory function.

3. THE PHYSICAL MECHANISM OF FIBROTIC REACTIONS

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(INTRODUCED BY WILLIAM S. MIDDLETON, M.D.)

Quartz, cholesterol, and tuberculosis produce fibrous reactions in tissue which differ quantitatively but not qualitatively in histology. This has been demonstrated experimentally by others. The symbiotic relationships between quartz and tuberculosis phospholipid have been repeatedly demonstrated.

Fibrous tissue reactions are currently considered as protective against toxins and poisons in solutions. It is pointed out that fibrous tissue is unqualified physiologically as a barrier against substances in solution. Fibrous elements are adapted to confine stress in the physical sense. In health and disease, fibrosis contributes physical support to the biologic economy.

The architecture of fibrous tissue reaction conforms with Newton's equations of force. This is demonstrated by slides of biologic preparations compared with force field preparations using magnets and iron filings.

Many irreconcilable objections are recognized by those who have reviewed the solubility theory of silicosis. These are enumerated. These objections are reconcilable with another basic nonchemical mechanism.

A search for physical properties common to fibrosive materials has revealed that there is a direct relationship between the fibrosive properties of a substance and its solid state molecular symmetry. Detailed reports are in press at the present time elaborating this point.

Those substances which exist in a state of molecular asymmetry compatible with piezoelectric reactions are observed to produce fibrosis. Piezoelectricity is a mechanism whereby mechanical and electrical energy states may be converted from one to the other in either direction. A more detailed definition of piezoelectricity is presented.

One phase of our present study is presented. Barium titanate was selected because of its spectacular piezoelectric properties, its lack of solubility, and the absence of elemental silicon in its chemical makeup. The preparation of animals is described and the results obtained are presented, with the use of slides and tables.

A reference is made to the scope of the present problem and reports now in press.

Conclusions.—(1) Fibrosive properties and piezoelectric properties of materials introduced into tissues are directly correlated.

2. The fibrosive potential of a material selected on the basis of its physical properties alone has been demonstrated.

4. THE EFFECT OF THE ANTICOAGULANT DRUGS UPON THE CORONARY FLOW

N. C. Gilbert, M.D., and Lester Nalefski, M.D. (By Invitation)
Chicago, Ill.

During the last few years the anticoagulant drugs, heparin and Dicumarol, have been shown to have a favorable effect upon coronary occlusion. Mortality has been greatly reduced and peripheral vascular accidents have been made much less frequent through the use of these drugs. It does not seem altogether reasonable, however, that these results are due to the anticoagulant action of these drugs alone. When the case reaches the attention of the physician, the occlusion and infarct are already an accomplished fact. Anticoagulant therapy cannot alter the already formed thrombus, whatever effect it might have upon future thrombotic processes. There is no definite evidence that the clot propagates or extends, except on rare occasions, and there is a great deal of evidence that the clot does not propagate. It has been shown also that many instances of occlusion are initiated by a subintimal hemorrhage. According to Wartman, these latter comprise at least 18 per cent.

Our own clinical results with coronary vasodilator drugs, such as aminophylline, followed by oral preparations of the xanthines, closely parallel the results obtained by others with anticoagulant therapy.

In experimental work upon animals and upon the empty beating heart, we have been able to show that the use of either heparin or Dicumarol increases the coronary flow volume. With heparin the effect varies from no effect to

a moderate effect, depending upon the preparation. Dicumarol, however, has a very definite effect upon the coronary flow. The effect is equal to that of the xanthines, and apparently lasts longer. We believe that the favorable effects resulting from the use of these drugs are primarily due to their action in increasing the coronary flow.

5. CORRELATION OF ELECTROCARDIOGRAPHIC AND PATHOLOGIC FINDINGS IN INFARCTION OF THE INTERVENTRICULAR SEPTUM

GORDON B. MYERS, M.D., HOWARD A. KLEIN, M.D. (BY INVITATION), AND TOMIHARU HIRATZKA, M.D. (BY INVITATION), DETROIT, MICH.

In a consecutive series of 161 patients with pathologically established myocardial infarction with Wilson precordial, standard and Goldberger limb leads, infarction of the interventricular septum was demonstrated in 102 patients and was accurately localized by means of coronary injection with radiopaque mass, roentgenogram, and multiple microscopic blocks. The cases were classified into three groups according to distribution of the pathologic lesion: (1) infarction largely confined to the septum, nine cases, (2) septal extension of anterior or anteroposterior infarction, sixty-nine cases, (3) septal extension of posterior infarction, twenty-four cases.

The electrocardiogram furnished suggestive or diagnostic evidence of septal infarction in all patients in group 1, in 80 per cent of group 2, and in only 33 per cent of group 3. This evidence was derived from the precordial leads in all but three instances in which it was obtained from aV_F. The standard leads were of no help. Diagnostic failures were prone to occur when the septal portion of the infarct was limited to the posterior half or to the apical third of the septum. The electrocardiographic findings were classified as follows:

1. Complete auriculoventricular block due to acute posteroseptal infarct was present in two patients.

2. QRS-T abnormalities diagnostic of infarction of the anterior portion of the septum were found in right precordial leads of forty patients. These comprised (1) right bundle branch characterized by an abnormal Q wave and/or elevated RS-T junction in $V_{1,2}$ in thirteen patients; (2) triphasic qrS complex of normal duration in $V_{1,2}$ in five patients; (3) QS complexes and upward RS-T displacement in leads over the right atrium, accompanied by abnormal Q waves in leads farther to the left, in twenty-two patients. QRS-T abnormalities in $V_{1,2}$ that were considered suggestive, but not diagnostic, of septal infarction were found in eighteen other instances. An abnormal QS or qrS complex referable to infarction of the posterior portion of the septum was recorded in lead aV_F as a result of horizontal cardiac position in seven patients.

3. Left bundle branch block was present in four instances, but was attributable to septal infarction in only one. Patterns characterized by QRS prolongation, an initial downstroke, and late intrinsicoid deflection in left axillary leads were present in ten patients, but were ascribed to subendocardial anterolateral infarction rather than the septal lesion.

4. Signs of anteroposterior infarction, considered presumptive of involvement of the intervening septum, were present in nine additional patients.

The correlation of electrocardiographic and pathologic findings will be brought out by case presentations.

6. BEDPAN DEATHS

JOHNSON MCGUIRE, M.D., ROBERT GREEN, M.D. (BY INVITATION), SANFORD R. COURTER, M.D. (BY INVITATION), JEAN NOERTKER, A.B. (BY INVITATION), ARNOLD IGLAUER, M.D., VIRGIL HAUENSTEIN, M.D., AND JOHN BRAUNSTEIN, M.D. (BY INVITATION), CINCINNATI, OHIO

The notorious frequency of sudden and unexpected deaths of patients while using bedpans in hospitals has been commented upon for many years.

In an attempt to discover the factors responsible for such deaths, the autopsy protocols and clinical records of patients in the Cincinnati General Hospital who died on bedpans and were autopsied have been reviewed for the ten-year period from 1936 to 1946, inclusive.

A second approach to the problem of determining the causes of death in such cases has been the measurement of alterations in circulatory dynamics accompanying simulated efforts of normal individuals and patients with organic heart disease to move the bowels.

The precise mechanism of death in the twelve cases examined at postmortem (all with organic heart disease) was determined in only three instances: (1) ruptured aortic valve, (2) pulmonary embolism, (3) ruptured dissecting aneurysm.

Intravascular pressures measured by strain gauges and graphically recorded in normal subjects and patients demonstrated marked rises of pressure during the initial phase of bearing down within the femoral artery, left ventricle, pulmonary artery, right ventricle, right auricle, and peripheral veins. Toward the end of the strain a fall in blood pressure occurred; subsequently a marked rise of pressure developed.

Electrocardiographic tracings in twenty-five normal subjects during strain showed first bradycardia, then marked tachycardia. Following cessation of strain, marked bradycardia developed. Migration of the pacemaker and premature contractions occasionally were noted. Patients with organic cardiovascular disease showed few such changes.

Since the cause of death frequently is not found at autopsy, it is believed that cardiac arrhythmias or cardiac standstill may be the usual cause of bedpan deaths.

Measures to combat such deaths are suggested.

7. EMETINE THERAPY IN THE PRESENCE OF HEART DISEASE

W. A. SODEMAN, M.D., NEW ORLEANS, LA.

Electrocardiographic changes occur in 25 to 50 per cent of patients with normal hearts under emetine therapy, and the drug is generally considered contraindicated in the presence of heart disease. We have studied eight patients in whom advanced heart disease was present and to whom emetine was administered because hepatic amebiasis was a greater threat to life than was the heart disease. In five, closed drainage also was performed. All were at bed rest. Five had arteriosclerotic heart disease, two being at the time under digitalis therapy. One had, in addition, hypertension and known previous congestive heart failure. One had congenital heart disease with interventricular septal defect and bundle branch block; another, rheumatic heart disease with mitral stenosis. Six showed abnormal electrocardiograms before emetine therapy was started. Under a controlled regimen of treatment, with emetine dosage based upon body weight (1 mg. per kilogram body weight daily, not to exceed ten days), none of the pa-

tients developed symptoms or signs of heart failure. Only one showed additional electrocardiographic changes attributable to emetine. One death occurred three months and another one year following therapy, both from other known causes. In all patients the amebiasis was effectively controlled.

Despite usual warnings that emetine should not be administered to cardiac patients, dangers from hepatic amebic disease at times outweigh those that may result to the heart from emetine and treatment should be instituted. The added risk in the use of emetine under these circumstances, when its need is great, must be accepted. Adjustment of dosage schedules to fall within 1 mg. per kilogram body weight daily for ten days was, in the eight patients cited, effective for the amebiasis and was accompanied by no demonstrable adverse effects on the already damaged cardiovascular system. The use of emetine in the presence of heart disease is discouraged except when necessary for forms of amebiasis endangering the patient's life and not effectively treated otherwise.

8. THE PRESIDENT'S ADDRESS

9. THE PATHOGENESIS OF ACUTE BACTERIAL LYMPHADENITIS

RALPH O. SMITH, M.D. (BY INVITATION), AND W. BARRY WOOD, JR., M.D.
ST. LOUIS, MO.

In an effort to clarify the role of lymphatic tissue in antibacterial defense, a study has been made of the cellular reactions in popliteal lymph nodes of rats inoculated in the foot pads with Type 1 pneumococcus.

Acute pneumococcal lymphadenitis is characterized by rapid infiltration of polymorphonuclear leucocytes into the intermediary sinuses of the node and prompt phagocytosis by both the macrophages of the sinuses and the recently arrived leucocytes. By seven hours the polymorphonuclear leucocytes are found densely congregated about the hilar region, and nine hours after inoculation the phagocyted organisms have been digested and pneumococci are no longer seen in the node. At the end of twenty-four hours the node presents the picture of a subsiding inflammation with a marked macrophage reaction and regenerating follicles.

By eradication of hilar blood supply, direct intralymphatic injection of pneumococci, and analysis of cells in afferent lymph, it has been shown that the majority of polymorphonuclear leucocytes entering the intermediary sinuses come from capillaries lining these sinuses, whereas the leucocytes present in the subcapsular sinus come from the primary inflammatory focus in the footpad as well as from capillaries of the capsule and the subcapsular portions of the follicles.

Phagocytosis of pneumococci in the foot pad and popliteal node occurs in less than thirty minutes after inoculation. Because of the promptness with which the phagocytic reaction takes place, and because of the large surface area afforded the leucocytes by the nodal sinuses and interstitial tissues of the foot pad, it is assumed that the same nonantibody mechanism of "surface phagocytosis" is involved as that previously described in experimental pneumonia.

Fibrin formation in the sinuses of the node is rare. This finding may be related to the observation that five minutes after inoculation, mast cell granules, which are known to contain herapin, are strewn throughout the sinuses of the node. The mast cells become vacuolated and almost devoid of granules; later their granules appear to regenerate.

These studies indicate that, early in the course of acute infection, bacteria reaching a regional lymph node elicit a prompt leucocytic response in the node.

The resulting enhancement of filtration due to the congregation of leucocytes in the hilar sinuses and the immediate phagocytosis of bacteria in the absence of opsonin lead to rapid destruction of invading organisms. Thus an acutely inflamed lymph node, so frequently regarded as a passive filter, in reality plays an active role in antibacterial defense.

10. ANTIBODY FORMATION IN HUMAN SUBJECTS FOLLOWING THE INGESTION OF HEAT-KILLED BRUCELLA ABORTUS

ABRAHAM I. BRAUDE, M.D., AND DAVID GOLD, B.S., MINNEAPOLIS, MINN.

(INTRODUCED BY WESLEY W. SPINK, M.D.)

The agglutination test is used widely in the diagnosis of brucellosis. Caution must be exercised in its interpretation, however, because agglutinins are known to appear in the sera of persons without brucellosis. Eisele and McCullough have found that positive Brucella agglutination tests develop following cholera vaccination. This probably accounts for only a small percentage of false positive reactions. At the University Hospitals, Aagaard has observed that Brucella agglutinins were present in 9.3 per cent of blood samples obtained from consecutive rural patients who were being studied in a dispensary service for various cases. The following investigation was undertaken to determine whether the repeated ingestion of nonviable Brucella organisms in milk would produce demonstrable agglutinins in the serum.

A preliminary study disclosed that no agglutinins appeared in the sera of guinea pigs following the oral administration of 100 billion heat-killed *Brucella abortus* organisms in single or divided dosages. The same dose was then given in aqueous suspension to twenty-three human volunteers in a single feeding and it was found that serum agglutinins had developed in four subjects. An attempt was made next to approximate the naturally occurring circumstances whereby Brucella enter the gastrointestinal tract in pasteurized milk. This was accomplished by the daily feeding to thirty-six hospital patients of 100 million heat-killed Brucella organisms in pasteurized milk obtained from Brucella-free herds. After ten to twenty feedings, during periods of two to four weeks, significant titers of agglutinins were detected in eleven of the thirty-six persons. When the total number of ingested Brucella was reduced to one million daily, it was found that one of eight additional patients showed a rise in titer. In a total of sixty-nine individuals, agglutinins were produced in sixteen following the ingestion of nonviable Brucella under these varying conditions. Titers ranged from 1:20 to 1:640.

The ability of dead Brucella to sensitize the skin to Brucella antigens was investigated in eighteen persons who ingested 100 million organisms daily for ten to twenty feedings. Sixteen of the eighteen had negative skin tests with Brucellergen antigen after the feedings were discontinued. Two persons with positive tests at the beginning of the experiment showed no alteration in skin sensitivity as a result of the feedings. The sera of thirteen subjects in whom agglutinins for Brucella had appeared were also tested for opsonins before and after the ingestion of Brucella. In seven of these, there was an increase of ten to ninety over the initial opsonocytophagic index.

From these results it is concluded that the oral ingestion of nonviable Brucella may give rise to the production of agglutinins or opsonins but not to the acquisition of dermal hypersensitivity. This is offered as one explanation for the fairly high incidence of agglutinins in the blood of asymptomatic persons who inhabit areas where Bang's disease is prevalent in cattle. Under naturally

occurring conditions these dead organisms may be ingested in pasteurized milk or may result from the action of gastric juice on living organisms which enter the stomach in raw milk. Data have been accumulated demonstrating the marked anti-Brucella action of human gastric juice.

11. THE DEVELOPMENT OF STREPTOMYCIN-RESISTANT BACTERIA IN THE STOOLS OF PATIENTS TREATED FOR TUBERCULOSIS

MORTON HAMBURGER, M.D., JEROME R. BERMAN, M.D. (BY INVITATION), AND ANGELINA FABRIZIO, M.S. (BY INVITATION), CINCINNATI, OHIO

The development of streptomycin-resistant pathogens during streptomycin therapy has been demonstrated often enough to establish this phenomenon as a serious therapeutic and public health risk. However, no studies have been made of the effect of streptomycin therapy on bacteria which normally lead a saprophytic existence in the body but which are nevertheless potentially pathogenic. To investigate this problem, studies have been made of the streptomycin resistance of coliform bacilli in the stools of patients given streptomycin for various types of tuberculosis.

Samples of stool were obtained when possible prior to therapy, and at various intervals during and after therapy. Saline suspensions of the stool were streaked on each of two plates: an eosin-methylene blue plate and a similar plate containing 100 μg streptomycin per cubic centimeter. Growth was recorded after twenty-four, forty-eight, and seventy-two hours of incubation; photographs of the plates were made, and representative colonies were picked for more accurate determination of streptomycin resistance in a serial dilution test.

The study included seven patients with miliary tuberculosis, eleven with nonmiliary pulmonary tuberculosis, six with tuberculous peritonitis, and three with tuberculous pericarditis. All received 1 or 2 Gm. of streptomycin daily intramuscularly for one to twelve weeks, but no streptomycin by any other route.

Colon or aerogenes bacilli which grew freely in 156 to more than 2,500 μg streptomycin per cubic centimeter of medium appeared during treatment in the stools of six out of seven patients with miliary tuberculosis and in four of six patients with tuberculous peritonitis, but in no case of pericarditis and in only one of eleven cases of nonmiliary pulmonary tuberculosis. Sensitive strains from these patients were inhibited by 0.6 to 2.4 μg per cubic centimeter.

The ecologic relationship in the stool between sensitive and resistant variants was interesting. Though sometimes the resistant forms entirely replaced the sensitive strains during treatment, sensitive bacilli returned after cessation of therapy and usually ultimately completely replaced the resistant forms. This phenomenon was not a reversion of resistant to sensitive variants, nor a manifestation of "streptomycin-dependency," because resistant strains retained their resistance after more than 100 passages in streptomycin-free broth and after twelve passages through untreated mice.

In two patients with tuberculous peritonitis, resistant bacilli decreased in number or disappeared during the stage of clinical improvement while the patients were still receiving streptomycin.

12. BACTEREMIA FOLLOWING TOOTH EXTRACTION; PREVENTION WITH PENICILLIN AND 3,4-DIMETHYL-5-SULFANILAMIDE-ISOXAZOLE (GANTROSAN)

PAUL S. RHOADS, M.D., AND WARREN R. SCHRAM, D.D.S. (BY INVITATION)
CHICAGO, ILL.

WITH THE TECHNICAL ASSISTANCE OF DORIS ADAIR

The present study was undertaken with the following objectives: (1) To confirm the contention of other investigators that a temporary bacteremia can be demonstrated in a fairly high percentage of persons immediately after tooth extractions. (2) To learn, if possible, which teeth are most likely to cause bacteremia when extracted or traumatized. (3) To determine the effectiveness of penicillin and a new sulfonamide, 3,4-dimethyl-5-sulfanilamide-isoxazole (Gantrosan), in preventing the blood stream dissemination of bacteria.

One hundred twenty-two blood cultures were done immediately after teeth were extracted either because the teeth were infected or to prepare the gums for full dentures. In a group of twenty-eight the patients received intramuscular procaine penicillin in oil (300,000 units) two hours before extraction and the blood culture was positive in two (7.2 per cent). In a group of eight patients receiving 100,000 units of penicillin in aqueous solution two hours before tooth extraction there were no positive blood cultures. Eighteen blood cultures were from patients who received 2 Gm. of Gantrosan orally two hours before tooth extraction, and none of these were positive. Thus in the entire protected group the incidence of temporary bacteremia was 3.7 per cent. In the control group of sixty-eight in which the patients received no preoperative medication, 35.3 per cent of the blood cultures were positive for nonhemolytic streptococci.

Quantitative cultures were made of the snipped-off roots of the teeth extracted. The bacterial counts of the teeth removed in the protected and nonprotected groups were approximately the same. In attempting to correlate the roentgen appearance of the teeth with bacterial counts of the root ends and incidence of postoperative positive blood cultures, no constant relationship could be determined. Frankly abscessed teeth were associated with postextraction bacteremia a little more often than roentgenographically negative teeth, but in many instances bacteremia resulted from extraction of the latter, also.

The extreme importance of protecting persons with known valvular heart disease with penicillin or sulfonamide before tooth extractions is obvious. To be on the safe side the procedure should probably precede all tooth extractions.

PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 29, 1948

FRIDAY AFTERNOON, 2:00 P.M.

13. RELATIONS BETWEEN VOLUMES OF CLOSED HYPOTHERMAL CEREBRAL LESIONS AND SYMPTOMS IN RABBITS

C. Bruce Taylor, M.D. (By Invitation), George M. Hass, M.D., and John E. Maloney, M.D. (By Invitation), Chicago, Ill.

Acute closed cerebral lesions characterized by hemorrhage, necrosis, and progressive edema were produced hypothermally in rabbits without interrupting the continuity of the calvarium or introducing variables incidental to mechanical trauma. The dimensions and locations of lesions in the cerebrum were controlled so that they could be reproduced topographically and quantitatively in successive animals. Although hemorrhage, edema, and necrosis varied slightly in lesions which were otherwise identical, the variations were restricted to discrete volumes of injury.

When unilateral or bilateral lesions of the cerebrum occupied less than 9.4 volumes per cent of the brain, symptoms were negligible. When the lesions occupied 9.4 to 18.5 volumes per cent of the brain, severe symptoms developed in many animals. The data indicated that the minimum lethal volume of cerebral damage in 50 per cent of more than one hundred animals was 14.3 per cent of the volume of the brain. Severe clinical courses with an average postoperative duration of about seven hours and fatal termination always occurred when the lesions occupied more than 18.5 volumes per cent of the brain. A great majority of the animals that died had a normal postoperative period of behavior. Secondary lapse into stupor was a dependable indication of impending coma and eventual death within twenty-four hours after the time of completion of the operation.

These data indicate that a quantitative experimental approach to the problem of treatment of acute expanding closed intracerebral lesions characterized by local necrosis, hemorrhage, and edema can now be made. The postoperative duration of life can be predicted from the magnitude of the lesions which are produced and this duration is sufficient to permit evaluation of most therapeutic methods now used empirically in the treatment of acute expanding closed intracerebral lesions in human beings.

14. PARA-AMINOBENZOIC ACID THERAPY IN SCLERODERMA AND LYMPHOBLASTOMA CUTIS

Chris J. D. Zarafonetis, M.D., Ann Arbor, Mich.

(Introduced by Frank H. Bethell, M.D.)

Previous reports have dealt with the effects of para-aminobenzoic acid (PABA) in leucemia, lupus erythematosus, dermatomyositis, and dermatitis herpetiformis. The purpose of this communication is to describe the results of PABA therapy in patients with scleroderma and with lymphoblastoma cutis

The patients used in this study were made available by Dr. A. C. Curtis, Professor of Dermatology at the University of Michigan Medical School.

Five patients, presenting a wide range of sclerodermatous involvment, were treated with sodium (NaPAB) and/or potassium (KPAB) para-aminobenzoate. Improvement occurred in four and was most evident in the more extensively involved patients. The sclerodermatous areas gradually softened and became thinner with consequent increase in range of motion of the affected part. The fifth patient developed drug fever and therapy was discontinued.

Similarly, five patients with lymphoblastoma cutis were treated with NaPAB. All experienced relief from pruritis and objective improvement of the skin. This was characterized by diminution in erythema and infiltration. Treatment with NaPAB was discontinued in all five because of the development of edema. One of these patients was changed to KPAB therapy with loss of edema and striking improvement. Treatment was maintained for nine months in this subject. Of incidental interest has been the concomitant darkening during therapy of the patient's previously gray hair.

Administration of large amounts of PABA compounds results in glycosuria. This may not be verified in fermentation tests since the concentration of PABA in the urine of these patients will inhibit yeast fermentation activity.

All other forms of therapy for their respective disorders had been attempted in these patients before PABA therapy was undertaken. It would seem, therefore, that PABA has a practical, though limited, value in the treatment of scleroderma and lymphoblastoma cutis. More important, perhaps, is the possibility that further study of PABA may yield information as to the mechanisms involved in these and related conditions of unknown etiology.

15. THE INHIBITORY EFFECT OF NITROGEN MUSTARD (BIS BETA-CHLOROETHYL AMINE) ON THE DEVELOPMENT OF HUMORAL ANTIBODIES, CUTANEOUS HYPERSENSITIVENESS, AND VASCULAR LESIONS IN RABBITS FOLLOWING INJECTIONS OF HORSE SERUM

SAMUEL C. BUKANTZ, M.D. (BY INVITATION), GUSTAVE J. DAMMIN, M.D., KEITH S. WILSON, M.D. (BY INVITATION), AND MARY C. JOHNSON, A.B. (BY INVITATION), ST. LOUIS, MO.

The vascular lesions that appear in the rabbit following the injections of horse serum are similar to those observed in certain diffuse vascular diseases in man. The latter are believed by some to result from hypersensitiveness. Because of these similarities, the pathogenesis of the experimental lesions has been intensively investigated. The relationship of humoral antibody and cutaneous hypersensitiveness to the vascular lesions is still to be defined. Conflicting reports have been published concerning the influence upon the vascular lesions of various agents believed to affect antibody formation, antigen-antibody combination, or the cellular effects of the latter.

Antihistaminics were used in our initial attempt to modify the vascular lesions. Neither Benadryl nor Neohetramine was observed to affect the lesions, the development of antibodies, or cutaneous hypersensitiveness.

Because of the ability of sulfur and nitrogen mustards to suppress antibody formation (Hektoen and Corper, Phillips and co-workers and Spurr), we investigated the effect of *bis* beta-chloroethyl amine (HN_2) on the formation of antibody as well as on the development of cutaneous hypersensitiveness and vascular lesions.

A group of rabbits weighing 2.0 to 2.5 kilograms were given, at four-day intervals, a total of seven intravenous injections of 0.5 mg. HN_2 per kilogram. Two days after the third injection, each received 10 c.c. of horse serum per kilogram intravenously. A second group of rabbits received an identical dose of horse serum at the same time, but was not treated with HN_2. Two days after the seventh mustard injection and fourteen days after the serum injection, all animals were skin-tested with varying amounts of horse serum after which they were sacrificed. Blood serum samples obtained one week after injection of horse serum and again at the time of sacrifice were studied for the presence of horse serum antigen and antibody by precipitin tests. Pooled terminal serums of the two groups were separately analyzed quantitatively for total antibody nitrogen.

A marked leucopenia and moderate bleeding tendency developed in the HN_2 treated animals but they otherwise appeared healthy and gained weight. Antigen persisted the same length of time in the untreated as in the treated rabbits. Antibody appeared after one week in the control animals but not until the end of the second week in the mustard-treated group. In the pooled terminal serums of the control group 0.062 mg. antibody nitrogen per milliliter was present and but 0.031 mg. in the treated. There was moderate suppression of skin hypersensitiveness in the HN_2 treated group. Eighty per cent of the control group developed arterial and endocardial lesions whereas there were no vascular lesions observed in the treated animals.

While the data suggest that humoral antibody is important in the pathogenesis of the experimental vascular lesions, this has not yet been completely established, in view of the known effect of HN_2 upon protein structures and enzyme systems other than those involved in antibody formation. In this regard we have found that HN_2 in vitro had no effect upon the precipitation of rabbit antihorse serum by horse serum.

Summary.—The nitrogen mustard, *bis* beta-chloroethyl amine, was observed to suppress the development of humoral antibodies, cutaneous hypersensitiveness, and the vascular lesions in rabbits induced by horse serum.

16. EFFECT OF RUTIN UPON THE RESORPTIVE TOXICITY OF CERTAIN DRUGS

R. K. Richards, M.D., North Chicago, Ill.

Attempts to demonstrate an effect of rutin upon the capillaries by experimental methods have met with but limited success. Recently, reduction of trypan blue diffusion after local irritation of the skin of rabbits has been described (Ambrose and DeEds, J. Pharm. **90**: 359, 1947), and also the prevention of irradiation injury in rats (Griffith and co-workers, Proc. Soc. Exper. Biol. & Med. **64**: 332, 1947).

These procedures do not lend themselves readily to a quantitative measure of rutin action.

A new approach to this problem is based upon our early observation that sodium bisulfite in small concentrations (0.1 to 0.4 per cent) greatly increased toxicity of epinephrine or procaine if added to the drugs and injected intramuscularly or subcutaneously, but not intravenously. This "bisulfite phenomenon" was shown to be due to a local effect upon the capillaries by the bisulfite ion causing a faster absorption rate. Only drugs having a wide margin between the intravenous and the subcutaneous or intramuscular toxicity show a positive bisulfite phenomenon.

If rutin is injected intravenously in rats prior to the administration of the toxic drugs to which sodium bisulfite has been added the increased toxicity of epinephrine is greatly reduced without affecting the toxicity of epinephrine as such. This procedure permits a quantitative estimation of rutin action upon the capillaries.

A similar effect could be demonstrated against the increased toxicity of procaine caused by sodium bisulfite. However, in contrast to the observations with epinephrine, rutin also decreased toxicity of plain procaine solutions. It was shown that high concentrations of procaine exert a dilating effect upon capillaries which is antagonized by rutin.

Definite reduction of toxicity by previous rutin administration could also be shown with strychnine, but little, if any, with Metrazol. The former of these two drugs has a much greater margin between the intravenous and the intramuscular toxicity than the latter, thus the prolongation of the absorption produced by rutin permits the detoxification of the drug to become partially effective. The described procedures permit the use of bisulfite phenomenon, and the decrease of the absorptive toxicity of certain drugs, as a tool for further research on rutin on other drugs with specific effects upon the capillaries.

EFFECT OF SEDATIVES ON URINARY VOLUME OF PREGNANT WOMEN

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IOWA CITY, IOWA

(INTRODUCED BY W. M. FOWLER, M.D.)

One of the cardinal findings in toxemia of pregnancy is edema. In a previous communication before this Society, we reported the effectiveness of various diuretic agents in the mobilization and excretion of sodium and water. This paper reports our observations on the antidiuretic effect of morphine and other sedative drugs in pregnant women.

A series of patients was given a constant intravenous infusion for five hours and hourly urine specimens were obtained for eight hours. On test days these patients were given injections of a sedative, and the effect on the urinary output and chloride excretion was measured. Morphine reduced the urinary output by 50 per cent without any significant alteration in total urinary chlorides. The fluid retention was further manifested by a transitory increase in body weight.

Another series of patients was given oral fluids as a liquid breakfast of 1,000 c.c. during the hour of 7:00 to 8:00 A.M. The urine volumes were then measured at hourly intervals for four hours. On test days the sedative was administered one hour after the liquid breakfast. Morphine, Demerol, codeine, and Amytal caused similar depression in urinary volume. Paraldehyde and Avertin have not been found to have an antidiuretic effect.

DeBodo reported that morphine caused a release of the antidiuretic hormone from the pituitary in dogs. Studies were undertaken to determine the mechanism of this antidiuresis in women. The similarity of response by normal subjects and patients with diabetes insipidus to morphine suggests that this antidiuretic effect is not mediated through the posterior pituitary. Since hypnotic sleep did not induce this response, it is apparent that the antidiuresis was effected by some mechanism other than sleep itself.

These observations suggest that morphine and similar drugs should be used with caution in edematous states. Tables and graphs will present these data.

18. EVALUATION OF NEUROGENIC AND HUMORAL FACTORS IN BLOOD PRESSURE MAINTENANCE IN NORMAL AND TOXEMIC PREGNANCY USING TETRAETHYLAMMONIUM CHLORIDE

ALBERT A. BRUST, M.D. (BY INVITATION), N. S. ASSALI, M.D. (BY INVITATION), AND EUGENE B. FERRIS, M.D.
CINCINNATI, OHIO

Transient elimination of neurogenic tone by the action of sympathetic blocking agents has afforded a new physiologic approach to evaluation of mechanisms operative in sustaining blood pressure at normal or hypertensive levels. Tetraethylammonium chloride, (TEAC), administered intravenously in 400 mg. doses, induces a temporary blockade of impulse transmission at the autonomic ganglia. When neurogenic tone has been abolished in this manner, the arterioles remain responsive to humoral agents, and thus the remaining blood pressure (TEAC floor) must be maintained by humoral mechanisms together with intrinsic vascular tone.

Toxemia of pregnancy with its attendant hypertension has long been regarded as a humoral disorder despite the lack of physiologic evidence. In an effort to evaluate the relative importance of humoral and neurogenic factors in the blood pressure of patients with toxemia as well as those with normal pregnancy, we have utilized TEAC assay to study ten normal nonpregnant women, ten normal term pregnancies, eighteen cases of pre-eclampsia, and five cases of eclampsia.

In each of the normal term pregnancies, the prepartum response to autonomic block was a marked fall in blood pressure to mean levels of 55 to 65 mm. Hg. Within twenty-four to forty-eight hours post partum, all these patients showed a striking rise in TEAC floors, the responses corresponding exactly to those of the normal nonpregnant controls.

Strikingly different responses occurred in the patients with toxemia. Prepartum TEAC floors invariably remained elevated above those of nonpregnant controls and were consistently 25 to 70 mm. Hg higher than those of the normal pregnancies. With subsidence of toxemia post partum, floors promptly fell to the normal nonpregnant range. The diastolic TEAC floor appears to be of the greatest significance in these studies, for it never fell below 80 mm. Hg in toxemia yet was never higher than 56 mm. Hg in normal pregnancy. The highest diastolic floors consistently occurred in those patients who clinically were most toxic, regardless of the height of the pretest blood pressure.

The results suggest that: (1) The hypertension of toxemia of pregnancy is supported primarily by an excessive degree of humoral tone. (2) Conversely, the blood pressure in normal term pregnancy is maintained largely by neurogenic and intrinsic tone. (3) Within forty-eight hours post partum of normal pregnancy there is a return to nonpregnant control mechanisms (humoral predominating). (4) Clinical assay with TEAC may be a helpful aid in diagnosis of toxemia of pregnancy and in the evaluation of changes in severity during its course.

19. THE EFFECT OF WATER BY VEIN ON THE MORE SEVERE COMPLICATIONS OF CARDIOVASCULAR-RENAL DISEASE

F. R. Schemm, M.D., and J. A. Layne, M.D., Great Falls, Mont.

Our most seriously ill patients showed the greatest need for plain water and were the least able to take or retain adequate amounts by mouth. With a proper regulation of sodium intake, including a minimal use of plasma, blood, and other sodium-laden solutions which yield no free water, they were given plain water by vein in amounts designed to restore and maintain water balance.

The solutions used in about 500 periods of observation contained 50 or 25 Gm. of dextrose (quickly oxidized or stored) in 1,000 c.c. of distilled water which is quickly dispersed throughout the 50 liters of total body water. The usual volume given was 1,000 c.c., with a range of from 500 to 2,000 c.c. at one time. The total amounts given in one day ranged from a 500 c.c. supplement to from 3,000 to 6,000 c.c. daily when the oral intake was nil or negligible. The rate of flow was from 10 to 20 c.c. per minute. (It would have required too many hours to administer the larger totals at slower rates and it was noted that reactions occurred more frequently with the fatigue and annoyance of long-drawn-out venoclyses.) The effect of the larger amounts of fluid by vein was observed over periods of from five to thirty-five consecutive days.

There was no detectable clinical or laboratory evidence of harmful dilution observed immediately after a venoclyses or after several days of large amounts of these isotonic or hypotonic dextrose solutions. At the moment of completion of a venoclysis the changes in venous pressure were found to range from a drop of a few centimeters, through no change, to a rise of as much as 13 cm.; no objective or subjective evidence of left ventricular failure or pulmonary edema were noted with the rises in venous pressure. The venoclyses did not precipitate pulmonary edema in some cases which had recently experienced an acute profuse attack, and retinal edema and choked discs were seen to improve, and convulsions came under control during some periods of observations.

In severe myocardial infarction with shock, the water by vein was used effectively to prevent the development of anuria and azotemia, or for their correction. Severe congestive failure that had resisted sodium restriction and acid and mercurial diuretics was observed to respond to the increase in water intake made possible by intravenous supplements.

In many instances, what appeared to be an anuria with uremia due to so-called renal shut down proved to be an anuria due to an inadequate intake of plain water. For water became available to the kidneys and they elaborated urine readily again, only after a large existing plain-water deficit of from 4,000 to 7,000 c.c. and a high daily vaporization loss of 2,000 to 4,000 c.c. were provided for. In these patients the large amounts of plain water, given sometimes entirely by vein, did not induce heart failure and relieved the anuria and uremia without resort to decapsulation of peritoneal lavage.

20. THE NATURE OF THE ALTERED RENAL FUNCTION IN LOWER NEPHRON NEPHROSIS

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CHICAGO, ILL.

Serial determinations of clearances of mannitol, *p*-aminohippurate, urea, and creatinine, and of tubular excretory mass were carried out in three subjects in the phase of diuresis and recovery from lower nephron nephrosis. In addition, urine-plasma ratios of nonprotein nitrogen were followed in these and three other patients with lower nephron nephrosis during the whole course of illness. With these data it was possible to extrapolate back to the period of oliguria and recognize the general nature of the functional alteration.

In two subjects the first function tests gave negative values for tubular excretory mass and impossibly high values for filtration fraction and ratio of *p*-aminohippurate clearance to tubular excretory mass. In subsequent tests these distortions disappeared, but the absolute values for clearances remained lower than normal. Complete restitution of renal function to normal did not occur until three to seven months had elapsed.

At the beginning of diuresis, the concentration ratio of nonprotein nitrogen remained as low as during the oliguric period. After several days of diuresis, the concentration ratio began to rise steadily even as the urine volume increased, indicating improvement in tubular function.

These data are consonant with the idea that the functional renal lesion in lower nephron nephrosis is a diminished renal blood flow in association with a loss of specific function of the lower nephron. Consequently, the limited amount of modified glomerular filtrate reaching the lower nephron is almost completely resorbed. A gradient diffusion through the damaged cells apppears to be present which allows a greater resorption of *p*-aminohippurate than of mannitol and which is thus responsible for the distorted renal clearance tests. Recovery seems to be produced first by an increase in effective renal blood flow, followed later by a repair of tubular function. The gradient diffusion is apparently the first tubular defect to disappear. The total recovery of normal specific tubular function is much slower.

21. DANGERS FROM THE USE OF THE RICE DIET

LEO F. NARUT, M.D. (BY INVITATION), AND GEORGE C. TURNBULL, M.D.
EVANSTON, ILL.

The present public interest in the Kempner rice diet led us to make a critical evaluation of this diet as an adjunct to the treatment of hypertensive heart disease. The results of this and more liberal diets have been recorded in the treatment of fifty-seven patients with hypertensive cardiovascular disease on the medical services of the Cook County Hospital and the Evanston Hospital. Supportive case reports point out that a simple convenient criterion of the urine specific gravity tends to indicate that one group of patients is benefited by the rice diet, another is unaffected, while still a third is made worse. The organism as a whole must be considered; nor can one overlook the fact that fundamental physiologic and biochemical principles govern the potential efficacy of this type of diet. One must be mindful of the physiology of extra- and intracellular fluid, renal physiology, circulatory dynamics, and pulmonary ventilation; of the role of blood, psychosomatics, and the endocrine system; and of the influence of medication. Blood protein and sodium levels must be given due considera-

tion. Illustrative groups of patients having hypertensive heart disease are presented, showing the influence of the renal reserve as reflected in the urine specific gravity and the influence of a variable extrarenal environment. Twenty-five patients with a good renal reserve indicated by a urine specific gravity of 1.020 or better were benefited by the rice diet, especially where cardiac decompensation was present. The improvement noted is attributable to the results of weight reduction by dietary restriction. Twenty-four patients with a specific gravity range of 1.012 to 1.020 were possibly benefited by salt and protein restriction, but again to a limited extent, depending upon the status of the extrarenal environment. Eight patients with a specific gravity fixed at 1.010 were benefited at first but subsequently became worse when dietary restriction was continued. Serum sodium and chloride levels are altered; prerenal azotemia with sequela supervenes on a kidney with poor reserve; and clinically, the manifestations of weakness, lethargy, abdominal cramps, oliguria, and disturbances in acid-base balance become apparent. We consider urine specific gravity fixed at 1.010 as a warning signal to proceed with caution in the use of the rice diet, and we believe it imperative to keep in mind the various possibilities which may be encountered, especially in patients who have poor reserve in organs other than the kidney. When blood sodium levels begin to fall, the rice diet should be discontinued. The narrow border between the preuremic state and uremia necessitates the addition of salt and blood or fluids to the therapeutic regime in order to reverse the process and, thereby, possibly avoid a casualty.

22. THE EFFECTS OF BACTERIAL PYROGENS ON MALIGNANT HYPERTENSIVE PERSONS

ROBERT D. TAYLOR, M.D., A. C. CORCORAN, M.D., H. H. FERTIG, M.D. (BY INVITATION), AND IRVINE H. PAGE, M.D., CLEVELAND, OHIO

Thirty-five patients exhibiting the syndrome of malignant hypertension were treated with repeated injections of bacterial products which elicited leucocytosis and fever. The effects were evaluated from the levels of arterial pressure, cardiac, cerebral and renal functions, and funduscopic changes. The responses to all the materials were so similar that they can be considered together.

The diastolic blood pressure of sixteen of the thirty-five patients was reduced by treatment from 126 mm. Hg mean (range, 110 to 150) to average 100 mm. Hg during five to nineteen weeks. Papilledema disappeared in all but two, one of whom still is receiving treatment. The persistent elevation of the optic disc in the other may represent a lesion of the nerve. Fourteen patients became free of retinal hemorrhages. One of the remaining two is still under treatment, and, in the other, the severity grading was reduced from 4 to 1. Most of the fresh exudates disappeared; however, in nine persons scars of previous disease persisted.

Before treatment, five of these sixteen patients presented signs of congestive heart failure, all evidences of which were relieved during therapy. Electrocardiograms showing signs of damage became normal in seven and improved in the other nine. Heart size (teleroentgenogram) diminished from plus 18 per cent before to plus 5 per cent after treatment.

Renal function at first was moderately depressed in nine patients but was eventually restored to or above control levels.

Except for persistent elevation of arterial pressure, remissions have lasted for an average of twenty-four months and up to thirty months.

Eleven of the sixteen patients who responded well are living after fourteen to thirty-one months. Three of the remaining five discontinued treatment after

three to thirteen weeks and died within three months of cerebral hemorrhage. Another died of apoplexy ten months after treatment with pyrogen and six months after sympathectomy. The fifth, the only one with return of the malignant phase, died of cerebral hemorrhage six months after treatment was begun.

Of the remaining nineteen patients, eleven responded more briefly and in less degree than the sixteen described, while eight showed no change. All but two are dead. These two are at present under treatment.

Tolerance to pyrogens appeared in five to nineteen weeks, after which arterial pressure rose, but usually without reappearance of the malignant syndrome.

The outcome was not predictable from arterial pressure, the retinopathy, or the extent of cardiac disease. Six patients who were virtually blind and four who had congestive failure responded favorably. The significance of renal function is shown by the fact that TmPAH averaged 62 mg. per 1.73 sq. M. in the sixteen who responded well (range 48 to 109); the mean was 26 (range, 12 to 40) in those responding poorly, and 20.8 (range, 7 to 32) in those with no response.

In summary, the malignant phase of essential hypertension can be remitted in some patients by repeated injections of bacterial pyrogens provided renal damage has not advanced to the extent that TmPAH is less than 45 per milligram per minute per 1.73 sq. M. of body surface.

23. A TEST FOR THE PRESENCE OF THE HYPERTENSIVE DIENCEPHALIC SYNDROME USING HISTAMINE

HENRY A. SCHROEDER, M.D., AND MELVIN L. GOLDMAN, M.D. (BY INVITATION)
ST. LOUIS, MO.

A syndrome associated with arterial hypertension has been described by Page which he called the hypertensive diencephalic syndrome. Occurring usually in women, but also in men, it is characterized by labile hypertension and attacks of a periodic appearance of a blotchy rashlike blush on the face, chest and shoulders, associated often with headache, lacrimation, and signs of vasoconstriction in the extremities. Attacks of this disorder probably represent some neurogenic disturbance associated with hypertension. In susceptible individuals this syndrome has been reproduced by the intradermal injection of 0.25 mg. of histamine, reaching its height five to ten minutes after the injection. Of fifty patients with normal blood pressure, the syndrome was made to appear in five. Of thirty-three with neurogenic hypertension it appeared in twenty-six. In fourteen patients with other types of arterial hypertension it appeared in none. Four subjects in whom the reaction to histamine was marked failed to react after lumbodorsal sympathectomy. When the reaction occurred, the symptoms of which the patient complained usually were reproduced, including the typical hypertensive headache. Antihistaminic agents only partially blocked the reaction in one subject. It was not reproduced by the administration of epinephrine or Mecholyl. The possible role of histamine-like substances in causing some of the symptoms of neurogenic hypertension is suggested.

PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 30, 1948

SATURDAY MORNING, 9:15 A.M.

24. GLYCOSURIA SIMULATED BY THE ADMINISTRATION OF ASCORBIC ACID: ITS OCCURRENCE AND DIFFERENTIATION

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CINCINNATI, OHIO

The confusion afforded by repeatedly positive Benedict tests on urine of a diabetic patient whose diabetes was thought to be well controlled and who was receiving large doses of ascorbic acid prompted us to investigate the reducing power of this substance in the urine as measured by Benedict's qualitative test and the Clinitest tablet.

Quantitative in vitro experiments were performed in order to determine the amount of ascorbic acid which must be present in solution to give a positive Benedict test and Clinitest. It was found that 0.2 mg. of ascorbic acid was sufficient to reduce 5 ml. of Benedict solution to a 1 plus reaction and the Clinitest to a "trace"; 1.9 mg. of ascorbic acid produced a 2 plus with both tests, 2.1 mg. a 3 plus, while 8.0 mg. were required to give a 4 plus reaction. It was found that when compared by weight, ascorbic acid was a more powerful reducing substance than glucose when tested by these methods. It was further shown that ascorbic acid and glucose when present together in solution have an additive effect in this reduction reaction.

In vivo experiments showed that when ascorbic acid was administered in large doses parenterally (now a common surgical practice) or by mouth there was usually sufficiently rapid urinary excretion of this substance to give a positive Benedict test or Clinitest. Following the intravenous administration of 500 mg. of ascorbic acid the majority of the patients gave a 1 plus Benedict test in two hours and had a urinary output ranging between 38 and 136 mg. per cent ascorbic acid. Following the intravenous administration of 1,000 mg. of ascorbic acid, all but one of the patients had a 2 plus Benedict test after two hours. The urinary concentration of ascorbic acid ranged from 430 to 980 mg. per cent at that time.

A simple method depending on oxidation of ascorbic acid in alkaline medium has been devised for differentiating this substance from glucose in the urine. Two milliliters of urine are made alkaline with sodium carbonate followed by the addition of 6 to 8 drops of hydrogen peroxide. This procedure rapidly destroys the reducing power of ascorbic acid but does not interfere with the reducing power of glucose as measured by Benedict's test and the Clinitest. This procedure is useful not only in the differentiation between glycosuria and a false positive Benedict test due to ascorbic acid in routine urinalyses, but also enables one to follow more accurately the glycosuria of a diabetic patient who is receiving large doses of ascorbic acid.

25. "STEROID DIABETES" ASSOCIATED WITH CUSHING'S SYNDROME AND EXCRETION OF 17-HYDROXYCORTICOSTERONE (COMPOUND F) IN URINE; METABOLIC STUDIES

R. G. Sprague, M.D., Alvin B. Hayles, M.D. (By Invitation), H. L. Mason, Ph.D., M. H. Power, Ph.D., and W. A. Bennett, M.D. (By Invitation)
Rochester, Minn.

The diabetes which is commonly associated with Cushing's syndrome is probably explainable on the basis of an overproduction of carbohydrate-active steroid hormones by hyperplastic or neoplastic adrenal cortices. Such diabetes should, therefore, resemble the "steroid diabetes" produced by Ingle and coworkers in rats by the administration of 17-hydroxy-11-dehydrocorticosterone (Compound E) or 17-hydroxycorticosterone (Compound F); that is, it should differ from ordinary diabetes, particularly of the juvenile type, in being more resistant to insulin and in being mild during fasting. Furthermore, since one of the effects of the carbohydrate-active steroids is to stimulate the production of sugar from protein precursors, this type of diabetes should be characterized by the excretion of relatively large amounts of nitrogen in the urine, even when glycosuria is minimized by the use of diet or insulin, or both.

Observations which lend support to the foregoing ideas were made in the case of a 14-year-old boy with severe diabetes associated with Cushing's syndrome due to hyperplastic adrenal cortices. In addition to diabetes, the patient presented most of the classic features of Cushing's syndrome, including hypertension, osteoporosis, muscular weakness, and a hypochloremic, hypokalemic alkalosis of marked degree. That the diabetes was due to an overproduction of carbohydrate-active adrenal steroids was suggested by the fact that the urinary excretion of "cortinlike substances" ("11-oxysteroids") was remarkably high (about 17 mg. daily, or about 200 times normal). Indeed, 17-hydroxycorticosterone was isolated from the urine, 191 mg. of purified hormone being obtained from a twenty-five day collection of urine.*

Metabolic studies revealed that the diabetes exhibited the features of "steroid diabetes" in animals. It was of more than usual severity (in terms of insulin requirement), glycosuria being incompletely controlled with 130 units of insulin given daily while the patient received 187 Gm. of carbohydrate daily in the diet. Unlike the usual behavior in ordinary juvenile diabetes, however, the urine became virtually free of sugar (2.4 Gm. in twenty-four hours) and ketonuria was absent when food and insulin were withheld for twenty-four hours. (This amelioration of glycosuria with fasting and withholding of insulin was in marked contrast to the behavior of patients having both Addison's disease and diabetes who were previously studied by us, in whom withdrawal of insulin and fasting during the administration of Compound E resulted in the rapid development of severe diabetic acidosis.) As in the case of steroid diabetes in animals, nitrogen balance was decidedly negative with an intake of 57 Gm. Protein daily. Likewise, as suggested by the presence of osteoporosis, the balances for calcium and phosphorus were also negative.

The patient died after surgical resection of one hyperplastic adrenal gland. At necropsy pronounced hypertrophy and hyperplasia of the adrenal cortices, a small thymoma, a parathyroid adenoma (not hyperfunctioning), osteoporosis, and bilateral renal calculi were found.

The clinical behavior of the diabetes, the metabolic data, and the finding of large amounts of 17-hydroxycorticosterone (Compound F) in the urine seem

*Mason, H. L., and Sprague, R. G.: Isolation of 17-hydroxycorticosterone From the Urine in a Case of Cushing's Syndrome Associated With Severe Diabetes Mellitus, J. Biol. Chem. 175: 451-456, 1948.

to justify the conclusion that the patient had "steroid diabetes" analogous to that produced in animals by the administration of carbohydrate-active adrenal steroids.

26. ROLE OF THE ADRENAL CORTEX IN URATE METABOLISM AND IN GOUT

WILLIAM D. ROBINSON, M.D., JEROME W. CONN, M.D., WALTER D. BLOCK, PH.D. (BY INVITATION), AND LAWRENCE H. LOUIS, SC.D. (BY INVITATION)
ANN ARBOR, MICH.

Urate metabolism on a constant diet has been studied in three normal and one gouty subject during administration of approximately 50 mg. (Armour's Standard) of purified pituitary adrenocorticotrophic hormone intramuscularly daily, in divided doses, for five to ten consecutive days. By using a specific enzymatic method employing uricase to determine blood and urine urates, errors, inherent in the conventional colorimetric methods were avoided.

Following the administration of adrenocorticotrophic hormone to normal subjects, the urate excretion increased from 50 to over 100 per cent above base line values. This increase appeared during the first twenty-four hours and was well sustained, reaching its peak on the sixth, seventh, and ninth injection days, respectively, in the three subjects. It was accompanied by an increase in excretion of nonurate chromogen in all three, but this was irregular and not sustained during the injection period. There was an apparent decrease in blood urate when determined by direct colorimetric methods which are known to be influenced by other reducing substances but no significant change in the blood content of urate or of nonurate chromogen, as measured by the uricase method. On the first postinjection day there was a sharp drop in urinary urate excretion to near basal levels, followed by a definite increase on the second to fifth postinjection days. There was associated other evidences of a rebound in pituitary-adrenal cortical activity at this time.

The injection of desoxycorticosterone acetate in doses of 20 mg. daily to a normal subject for ten days did not produce any significant effect on urine or blood urate levels.

The chief differences in response of the gouty patient to adrenocorticotrophic hormone as contrasted to normal subjects were (1) absence of a significant increase in excretion of nonurate chromogen, (2) a sharp decrease in true blood urate to less than one-half of the base line level, so that loss from body fluids by simple increased renal excretion could account for one-half to three-fourths of urinary increase, as compared to normal subjects where only negligible quantities could be acccounted for on this basis, and (3) absence of any secondary increase in urate excretion during the postinjection period. Effects on blood sugar, glucose tolerance, glycosuria, urinary nitrogen, amino acid, and 17-ketosteroid excretion were similar to those seen in the normal subjects, except for the absence of any evidence of a rebound in hormonal activity during the postinjection period.

The gouty subject, who had been free of attacks for nine months preceding study, had a mild but definite attack on the third to fifth postinjection days, at a time when all data suggested a depression of adrenal cortical activity. Talbott and associates (1935, 1940) described cyclic changes in urate, electrolyte, and water excretion in patients with gout and related these changes to acute gouty attacks. Their data can be interpreted as reflecting cyclic changes in adrenal cortical activity, with attacks occurring during periods of decreased

function. A common denominator for many of the incidents which have long been recognized as capable of provoking acute attacks of gout is furnished by their ability to affect pituitary-adrenocortical activity.

27. INFLUENCE OF PHYSICAL FITNESS AND CONVALESCENCE FROM MAJOR SURGICAL PROCEDURES ON THE MAGNITUDE OF THE RESPONSES TO EXERCISE OF THE BLOOD AND URINE LACTATE

DAVID I. ABRAMSON, M.D., WARREN H. COLE, M.D., ROBERT W. KEETON, M.D., MAURICE GEPHARDT, M.D. (BY INVITATION), JOSEPHINE M. DYNIEWICZ, PH.C. (BY INVITATION), SOPHIE J. PRESLEY, M.D. (BY INVITATION), AND GEORGE LAVERS, M.D. (BY INVITATION)
CHICAGO, ILL.

The changes in the values of the blood and urine lactate produced by exercise on a treadmill were studied in a group of fifty patients, in various states of physical fitness, before and after herniorrhaphy or cholecystectomy.

Preoperatively, with the same exercise load (three minutes on the treadmill), the maximal lactic acid level attained in the blood and the total quantity in the postexertional urine specimen were found to be much greater in patients in poor physical condition than in those in excellent shape. Generally, similar differences were noted in the case of submaximal and maximal exercises, despite the fact that the respective periods on the treadmill for individuals in good physical condition were three to six times as long as for those in poor shape.

Postoperatively, one group of nineteen patients was placed at complete bed rest for from six to thirteen days. Except for some individuals in excellent physical condition, the patients showed much higher maximal blood lactate levels with exercise during convalescence than was observed preoperatively. Very rarely were the readings back to the preoperative level by the twentieth postoperative day. The changes in the quantity of lactate in the postexercise urine specimens followed the same trend, in some instances the values remaining elevated for as long as sixty days after operation. It is of interest that the lactate studies continued to show abnormal changes postoperatively long after the results of the tests of liver function and of circulatory and respiratory efficiency had returned to preoperative levels.

Other groups of patients subjected to the same operative loads were placed on regimens involving early ambulation. Except for occasional patients initially in poor physical condition, these individuals showed, during convalescence, smaller increases in maximal blood lactate levels and lactate content of the urine with exercise, as compared with the preoperative readings, than were observed for the patients subjected to a period of postoperative bed rest.

It is concluded that the magnitude of the blood and urine lactate changes produced by exercise is definitely influenced by the state of physical fitness of the individual. Furthermore, lactate studies are of value in the measurement of the length of convalescence following surgical operations and in the evaluation of various therapeutic regimens used to reduce this period.

28. PORTAL CIRRHOSIS OF THE LIVER: A CLINICAL AND FUNCTIONAL STUDY

WILLIAM E. RICKETTS, M.D. (BY INVITATION), JOSEPH B. KIRSNER, M.D., AND WALTER L. PALMER, M.D., CHICAGO, ILL.

Fifty cases of portal cirrhosis of the liver were studied by biopsy and tests of hepatic function. The fifty cases were divided into three groups:

Group I included fourteen patients free of symptoms, except one who had hematemesis and melena, but with physical findings suggesting the presence of cirrhosis such as hepatomegaly, splenomegaly, or collateral circulation of the abdomen. In two, the cirrhosis was first noted at laparotomy. The presence of cirrhosis was established by histologic examination. The alterations in hepatic function were minimal or absent; the bromsulfalein test was the most consistently abnormal.

Group II included twenty-three patients in whom signs and symptoms of moderate degrees of hepatic disease were present. Fibrosis and hepatocellular changes were noted histologically in all. Function tests in this group were uniformly abnormal. Repeated studies of hepatic function were made in nineteen of the group during the course of medical management. There was no significant change in two, improvement was marked in fifteen, and the test ultimately yielded normal results in two.

Group III included thirteen patients with marked jaundice and other symptoms of hepatic dysfunction requiring hospitalization. The function tests were markedly impaired in all. Fibrosis and severe parenchymal changes were noted histologically in ten of the thirteen. Four were admitted desperately ill and died soon after hospitalization. Subsidence of symptoms, marked improvement in hepatic function, and regeneration of the liver parenchyma were observed accompanying medical management in nine. The symptoms disappeared completely in six of these, two improved greatly, and one has improved and has no symptoms other than an acquired hemolytic anemia.

The following impressions were gained: (1) Abnormalities of hepatic function in portal cirrhosis depend upon the alterations of the parenchyma. (2) Fibrosis of the liver per se does not change the tests of liver function. (3) Portal cirrhosis may be present without detectable abnormality of hepatic function, and (4) portal cirrhosis is not necessarily a progressive disease.

29. SERIAL SERUM CHOLINESTERASE DETERMINATIONS IN LIVER DISEASE

L. J. VORHAUS, M.D., H. H. SCUDAMORE, M.D., G. J. GABUZDA, M.D., G. R. MOREY, M.D., M. A. MALONEY, M.D., AND R. M. KARK, M.D.
CHICAGO, ILL.

(INTRODUCED BY ROBERT W. KEETON, M.D.)

It has long been known that the cholinesterase activity of the serum is abnormally low in patients with disease of the liver and its determination has been suggested in the past as an index of liver function. However, because of the difficulty in performing determinations of serum cholinesterase activity, and because of the wide range of normal values obtained, such determinations have not become popular. The recent development by Michel of a simple potentiometric method for the determination of cholinesterase activity now makes

this measurement a practical clinical tool. The method measures the change in pH due to the liberation of acetic acid in the hydrolysis of acetylcholine by cholinesterase.

To date serum cholinesterase activity has been estimated in forty-two patients and serial determinations have been made in twenty-six of these patients with liver disease, as well as in four normal subjects. In patients with liver disease serial determinations have correlated well with changes in clinical status at times when a battery of currently accepted liver function tests failed to show significant changes.

Serial determinations are reported of the serum cholinesterase activity of seven patients with cirrhosis of the liver and three healthy controls living in a metabolic ward for periods of 70 to 240 days. Determinations were made three times weekly before, during, and after intravenous therapy with human serum albumin containing less than 0.3 Gm. of sodium in each 25 Gm. of albumin. The total doses of albumin ranged from 125 Gm. in a three-day period to 3,650 Gm. in three periods totaling forty-seven days. It was found that six of the seven patients with liver disease showed a significant rise in the serum cholinesterase activity together with subjective and objective clinical improvement. In all of these cases the serum cholinesterase activity began to rise toward normal levels before improvement was detectable by any other laboratory means employed. Results suggest that serial determinations of cholinesterase activity afford a more sensitive index to slight changes in hepatic function than any of the other tests used in this study (that is, cephalin flocculation, thymol turbidity and flocculation, BSP excretion, serum bilirubin, immediate and total, two-hour Watson urinary urobilinogen, prothrombin time, total proteins with A/G ratio).

Studies are in progress on the rate of regeneration of serum cholinesterase activity following its destruction by di-isopropyl fluorophosphate. In thirteen patients with liver disease, as well as in four healthy controls, the return to serum cholinesterase activity to pretest levels has taken approximately four weeks, the healthy controls starting at and returning to a higher value than the patients.

30. ANTIDIURETIC ACTIVITY OF URINE IN ACUTE HEPATITIS

J. L. Sims, Madison, Wis.

(Introduced by O. O. Meyer, M.D.)

This study was undertaken in an attempt to confirm the presence of an antidiuretic factor in the urine of patients with liver disease and to elucidate the mechanism of its appearance.

Daily twenty-four hour urine specimens were obtained from nine patients with acute infectious hepatitis, one with serum hepatitis, and one with an acute febrile and icteric exacerbation of a portal cirrhosis with ascites and edema.

Protein precipitation at pH 4.0 and 80° C. was carried out; the supernatant was dialyzed twenty-four hours against tap water and evaporated to 100 ml. in cellophane sacks before an electric fan.

Assay was by intraperitoneal injection of 0.5 ml. per 100 Gm. body weight into groups of four adult male albino rats hydrated to 5 per cent of their body weight by tap water gavage. Controls received tap water or similar extracts from normal urines intraperitoneally. Urinary output per 100 Gm. of body weight was plotted for three- or five-hour periods and compared with the intake output records and clinical appearance of the patients.

In the early phases of acute hepatitis there was a sharp inhibition of diuresis in the assay animals which paralleled clinical evidences of a tendency to water retention. With beginning convalescence this inhibitory activity lessened and the output of assay animals approached or exceeded the controls. In two instances the first specimen obtained from a patient failed to show antidiuretic activity, although subsequent ones during the early phase of illness did. Similar results were seen over a longer period in the cirrhotic patient.

Samples of varying activity were assayed on male albino rats made cirrhotic by prolonged twice weekly injections of carbon tetrachloride. The degree of inhibition of diuresis in this group did not exceed that in simultaneously run normal control animals, and indeed, in about two-thirds was slightly less.

Five- and eight-hour urine outputs from both the control and cirrhotic animals were extracted in similar fashion, being evaporated to 12 ml., and reassayed. These preparations showed no increase in antidiuretic activity as compared with similar recoveries from animals injected with tap water.

These studies confirm Labby's findings of the presence of a factor showing antidiuretic activity in the urine of patients in the early phases of acute hepatitis. They do not support the thesis that failure of hepatic inactivation of a normally formed principle of this nature is the basis of its presence.

31. VITAMIN B_{12} IN PERNICIOUS ANEMIA AND PUERPERAL MACROCYTIC ANEMIA

FRANK H. BETHELL, M.D., MURIEL C. MEYERS, M.D. (BY INVITATION), AND ROSALIE B. NELIGH, M.D. (BY INVITATION), ANN ARBOR, MICH.

Four patients with pernicious anemia in relapse were treated with vitamin B_{12}* for periods of ten to twenty weeks. Intramuscular injections of 1 μg of B_{12} were given daily during the period of the reticulocyte response with subsequent treatments at varying intervals up to twenty-five days. Doses were adjusted so that the equivalent of 1 μg per day would be received. Three of the patients had maximum reticulocyte levels and rates of red cell increase which exceeded the expected response following daily injections of 1 U. S. P. unit of liver extract. The fourth patient received 1 mg. of the folic acid antagonist, Aminopterin,† daily for two days prior to B_{12} administration and for the first fourteen days of treatment with B_{12}. The reticulocyte response was delayed and suboptimal. When aminopterin was discontinued a second reticulocyte response occurred with rapid elevation of red cell count. All four patients attained normal hematologic values after six to eight weeks.

Three patients had nervous system involvement. Following B_{12} therapy there was relief of paresthesias and decrease in ataxia. Glossitis was troublesome in three patients and disappeared promptly on institution of treatment.

A girl aged 19 years was seen two months after delivery of a normal infant. The presenting features were severe anemia, intense glossitis, stomatitis, and vulvovaginitis. For at least two years she had received an extremely poor diet. Red cell count was 1,500,000, with macrocytosis, leucopenia, and megaloblastic marrow. Gastric contents contained hydrochloric acid. Intramuscular injections of 1 μg of B_{12} for ten days failed to produce hemopoietic or clinical responses. Anemia and leucopenia became more severe, and mucous membrane lesions progressed. Megaloblastic marrow reaction persisted. Subsequent treatment with folic acid, 10 mg. daily by mouth, was followed by conversion of

*Merck & Co., Inc.

†Lederle Laboratories, American Cyanamid Co.

marrow picture, reticulocyte response, increase in erythrocyte and leucocyte values, and rapid subsidence of stomatitis and vulvovaginitis.

Assays of feces of four patients with untreated pernicious anemia revealed high contents of growth-stimulating factor for *Lactobacillus lactis* Dorner, the test organism for vitamin B_{12}.* The content of B_{12} equivalent ranged from .3 to 1.8 μg per gram of dried feces. Thus the daily output of B_{12} by patients with pernicious anemia appears to be many times greater than that necessary to produce remission when introduced parenterally. This suggests that in pernicious anemia there may be defective absorption of vitamin B_{12} derived either from dietary sources or by intestinal bacterial synthesis.

32. STUDIES ON GLOBIN AND PORPHYRIN METABOLISM MADE WITH C^{14} AND N^{15}

MOISES GRINSTEIN, PH.D. (BY INVITATION), M. D. KAMEN, PH.D. (BY INVITATION), AND CARL V. MOORE, M.D., ST. LOUIS, MO.

Three different forms of tracer-tagged glycine have been used to label hemoglobin. Shemin and Rittenberg employed glycine containing N^{15} to demonstrate that this amino acid serves as a nitrogenous precursor of protoporphyrin; a small amount of the N^{15} was also incorporated in the total cell protein. They followed the N^{15} content of heme carefully and found that (a) the labeled protoporphyrin within red cells does not participate in the dynamic metabolic state, (b) is not re-utilized for heme formation when red cells are destroyed, and (c) serves as a reliable index of the length of life of the erythrocyte. Altman and associates, using methylene labeled glycine (C^{14}), have shown that the alpha carbon of glycine is utilized for the biosynthesis of hemoglobin protoporphyrin; a relatively small amount of C^{14} is incorporated into globin. When glycine labeled with C^{14} in the carboxyl position is fed to animals, the C^{14} is built into globin but not into protoporphyrin (Grinstein and co-workers). The first and last of these three methods of tagging hemoglobin were used in the experiments described below to study globin and porphyrin metabolism.

Glycine containing C^{14} in the carboxyl group was fed to two dogs and to one rat. In each instance, the C^{14} appeared in the globin portion of hemoglobin but could not be demonstrated in the heme. The concentration in globin rose rapidly in the dogs during the first few days, remained relatively constant until the sixty-fifth and eightieth days, respectively, and then fell to very low levels. The shape of the curve obtained was very similar to that found by Shemin and Rittenberg for heme labeled with N^{15}. The results indicate that the globin of intact erythrocytes (1) remains within the cell during its life span without participating in the dynamic protein interchange characteristic of nucleated cells and (2) is apparently not utilized again for new hemoglobin formation once the cell is destroyed.

In the second series of experiments, 5.6 Gm. of glycine tagged with 27.7 per cent excess N^{15} was fed to one dog which had previously been made anemic by bleeding. From blood obtained at intervals during the following thirty-four days, crystalline protoporphyrin 9 dimethylester was isolated and found to be tagged with the isotope (0.72-0.96 atom per cent excess N^{15}). During the first twenty days of the experiment, coproporphyrin I (tetramethylester) was isolated from the urine and feces of this animal; it contained N^{15} in a relatively high concentration (1.1 atoms per cent excess). On the thirty-fourth day, the dog was bled to death, and the total volume of red blood cells removed was trans-

*Performed by Dr. O. D. Bird, Parke, Davis & Co.

fused into a second anemic dog. The following day, crystalline protoporphyrin 9 isolated from the blood of the recipient animal contained 0.40 atom per cent excess N^{15}. An acute anemia was then induced with phenylhydrazine. Coproporphyrin I isolated from the combined urine and feces during this period had no demonstrable N^{15} (less than 0.03 atom per cent excess), while the protoporphyrin 9 dimethylester isolated at the same time from urine and feces did contain the isotope (0.133 atom per cent excess). On the twentieth day, when the animal was recovering from the phenylhydrazine anemia, protoporphyrin 9 dimethylester crystallized from its blood had N^{15} in a concentration of 0.132 atoms per cent excess. This experiment has not yet been repeated. Conclusions, therefore, must be regarded as being *preliminary*. The data suggest that coproporphyrin I is formed as a by-product during the biosynthesis of hemoglobin and is not derived from hemoglobin catabolism. This result was expected. More surprising, however, was the increased concentration of N^{15} in the protoporphyrin 9 from the excreta of the recipient dog, a finding which suggests that the excreted protoporphyrin 9 might, in phenylhydrazine anemia at least, be a derivative of hemoglobin degraded during the hemolysis. Free erythrocyte protoporphyrin of the red cells would be another possible source of the fecal protoporphyrin 9.

33. THE COAGULATION DEFECT IN THROMBOCYTOPENIC PURPURA

ARMAND J. QUICK, M.D., JACOB N. SHANBERGE, M.D. (BY INVITATION), AND MARIO STEFANINI, M.D. (BY INVITATION), MILWAUKEE, WIS.

In thrombocytopenic purpura the platelets are reduced, the bleeding time is prolonged, clot retraction is absent, spontaneous hemorrhages occur, and petechiae and other manifestations of vascular fragility appear. The coagulation time characteristically is nearly always normal. This has led to the tacit acceptance that no true coagulation disturbance is present in this disease and that platelets are of secondary importance in the coagulation mechanism.

Recent findings concerning the platelets makes it possible to offer a new concept of coagulation and thereby to formulate a better explanation for the basic hemostatic dysfunction in thrombocytopenic purpura. It is now certain that platelets are indispensable for coagulation. When these cells disintegrate they liberate an enzyme which immediately acts on the precursor of thromboplastin (thromboplastinogen), converting it to its active form. This product acts on the prothrombin complex to form thrombin. When platelets are diminished, the conversion of thromboplastinogen is delayed and therefore the activation of prothrombin is abnormally slow. This is easily measured by the prothrombin consumption test, which furnishes a better estimate of the true coagulability than the coagulation time since the latter is merely an empirical measurement of the time required for the formation of enough fibrin to meet an artificially set standard such as sufficient solidity to hold the blood when the test tube is inverted.

The prothrombin consumption is markedly reduced in severe thrombocytopenic purpura. The coagulation time may, however, even be shorter than normal and therefore mask the basic coagulation disturbance. In hemophilia the prothrombin consumption likewise is very low. Thus, the basic defect, inadequate formation of thrombin, is common to both hemophilia and thrombocytopenic purpura. Yet the clinical manifestations of these two diseases are distinctly different. One finds, moreover, occasionally a persistent low platelet count without a concomitant spontaneous hemorrhagic diathesis and a prolonged bleeding time. In is likely, therefore, that the characteristic features of

thrombocytopenic purpura such as the petechiae, the mucous membrane oozing of blood, and the prolonged bleeding time are not due directly to the deficiency of platelets but are caused by a factor affecting the vascular structure, particularly the capillaries, and this is superimposed upon the basic coagulation defect. In summary, the poor prothrombin consumption and the defective clot retraction are due directly to lack of platelets, while the spontaneous hemorrhagic tendency is attributable to a vascular factor whose action is accentuated by the coagulation defect.

34. A STUDY OF THE PLASMA DEFECT IN PATIENTS WHOSE BLEEDING IS TEMPORARILY CONTROLLED BY PROTAMINE SULFATE OR TOLUIDINE BLUE

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The anticoagulant action of heparin is rapidly overcome upon addition of protamine (salmine sulfate). Normally when a standard amount of heparin is added to a constant volume of blood (0.09 mg. of liquid heparin per milliliter of blood), its action is regularly overcome and clotting occurs with a standard amount of protamine sulfate, 0.120 mg. In certain hemorrhagic states, most but not all of which are associated with thrombocytopenia, considerably more protamine may be required before clotting occurs. These patients generally have a prolonged Lee-White clotting time, the clotting of which is preceded by a gel-like formation. This syndrome has been observed in patients with leucemia who are bleeding, patients under extensive nitrogen mustard and/or P^{32} therapy, a small percentage of patients with idiopathic thrombocytopenic purpura, in three patients who received heavy "spray" x-radiation, in some patients who bleed abnormally but in whom the platelet count and other known clotting studies are normal, and in certain patients with menorrhagia.

The nature of the defect is not known. It may be due to the release of heparin or heparin-like substances since the defect measured in this heparin protamine titration procedure may be temporarily corrected by the administration of protamine sulfate or toluidine blue. The possibility of a decrease of heparin co-factor associated with a heparinemia (?) has been explored. This possibility was suggested by the fact that the amount of heparin required to alter the heparin-protamine titration in normal patients or dogs renders the Lee-White clotting time incoagulable. This is in contrast to the defect seen in the patient with this bleeding syndrome whose clotting time may be only moderately prolonged but whose heparin-protamine titration is remarkably altered. However, since in vivo rapid intravenous heparin (0.25 to 0.50 mg. per kilogram) administration in such patients or in irradiated dogs has a much greater effect upon the whole blood clotting time than usually occurs, it appears unlikely that the heparin co-factor is abnormal.

Since many of the plasma and tissue proteins are antiheparins, it may be that the associated protein changes in some of these disorders in part account for the disturbance through the release of heparin or heparin-like substances, which may then affect the clotting system. Present studies suggest that a diminution in the antiheparins of plasma and/or an increase, relative or actual, of the plasma heparinoid substances are the systems most likely affected. More data are required before a final conclusion can be drawn.

35. CHANGES IN BLOOD AND BONE MARROW IN ACUTE LEUCEMIA INDUCED BY AMINOPTERIN

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Rochester, Minn.

(Introduced by F. J. Heck, M.D.)

4-Aminopteroyl glutamic acid (Aminopterin) was administered to eighteen adults and children with acute leucemia diagnosed by study of the blood and bone marrow. Remissions in the clinical course of the disease as well as improvement in the blood and bone marrow were observed in some instances, although many patients made no response to the drug. Toxic manifestations included stomatitis, diarrhea, alopecia, deafness, and temporary aplasia of the bone marrow. In an attempt to prevent these toxic effects, vitamin B complex, crude liver, and folic acid were used.

When the response to treatment was favorable, changes in all the elements of bone marrow were conspicuous. The myeloid cells assumed a more normal pattern and in lymphatic leucemia the lymphocytes decreased and became more mature. The increase in erythropoiesis was striking. When folic acid or liver extract was not administered, the marrow sometimes contained megaloblasts and hyperlobulated polymorphonuclear leucocytes. Liver or folic acid prevented megaloblastic changes or caused them to disappear after they were established.

These observations are further confirmation of the theory that Aminopterin has an action antagonistic to that of folic acid. The length of time over which the clinical observations have been made does not permit any definite conclusions about the eventual effect of Aminopterin in acute leucemia.

ADDITIONAL ABSTRACTS

36. THE MINIMAL SODIUM DIET. A CONTROLLED STUDY OF ITS EFFECT UPON THE BLOOD PRESSURE OF AMBULATORY HYPERTENSIVE SUBJECTS

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The administration of a diet rigidly restricted in sodium may influence the habits and reactions of the patient profoundly, altering blood pressure through mechanisms unrelated to the effect of sodium restriction per se. A controlled evaluation of the factors attendant upon the use of this diet is necessary, particularly in the nonhospitalized patient. Accordingly, twenty-one hypertensive subjects were placed on a calorically adequate diet estimated to contain less than 300 mg. of sodium per day and were followed for approximately eighteen weeks. Blood pressures were obtained in the clinic by a constant technique, and twenty-four hour urine collections were made once a week. All subjects received medication, consisting either of 4 Gm. of NaCl a day or of identical appearing placebos. After each six-week period the medication was either changed or continued according to prearranged schedules, initially selected at random for each subject by the pharmacist.

The experiment thus consisted of three periods, differing only in that during one (or two) period(s) supplemental sodium chloride was administered, while in two (or one) period(s) the sodium intake remained at a bare minimum. The subject, whose cooperation was solicited, was unaware of the nature of the medication, and during the study the investigators did not know which medication was being taken.

Results.—Data from which evaluation of the effect of rigid sodium restriction upon blood pressure could be based were available from only eight of the subjects. The criteria for their selection required that the twenty-four hour urinary sodium average below 500 mg. for at least one period and over 1,000 mg. for at least one period. For these subjects the average of the blood pressures taken during rigid sodium restriction was lower than the average during the periods of added sodium, by 4.72 mm. Hg diastolic (range, 18.5 to −8.02) and 4.96 mm. Hg systolic (range, 22.15 to −7.90).

All twenty-one subjects yielded data for at least two periods of a *like order* of sodium excretion, from which the variances of the blood pressures for the group were determined. On this basis, the probability that the experimentally observed differences could be due to chance is less than 1 in 50 for the diastolic values ($t = 3.01$) and less than 1 in 20 for the systolic values ($t = 2.36$).

Conclusions.—

1. A diet rigidly restricted in sodium is difficult to administer successfully to ambulatory hypertensive subjects.
2. A difference of less than 5 mm. Hg in average blood pressure was observed, ascribable to the effect of NaCl restriction alone.
3. This difference is statistically significant.

37. THE EFFECT OF DESOXYCORTICOSTERONE ACETATE AND PROPYLENE GLYCOL IN EXPERIMENTAL RENAL HYPERTENSION

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Experiments performed to determine the blood pressure response in renal hypertensive and normal dogs revealed the following: (1) a small but definite pressor effect from intravenous administration of propylene glycol in unanesthetized dogs, (2) no additional effect from intravenous administration of desoxycorticosterone acetate dissolved in propylene glycol, (3) no pressor effect from intravenous administration of desoxycorticosterone glucoside, and (4) a similar and sustained pressor effect from continued subcutaneous administration of desoxycorticosterone acetate in sesame oil in both normal and hypertensive dogs.

Initial experiments were performed on mongrel dogs trained for blood pressure determinations by femoral arterial puncture. Three were made hypertensive by renal arterial clamps or perirenal silk wrappings. Approximately eighteen months were allowed as the postoperative stabilization period. Desoxycorticosterone acetate in daily doses of 0.15 to 1.28 mg. per kilogram of body weight was given for a total of eight periods of six weeks each in three hypertensive and two normal dogs. Blood pressure rises of 25 to 50 mm. Hg were observed in seven instances. These were sustained and disappeared within two to four weeks of withdrawal of the drug. Repeat courses of the drug in the same animals produced shorter and less-pronounced elevations of blood pressure, which tended to return to control level before the drug was discontinued. In some instances the post-treatment levels were 20 to 30 mm. Hg lower than control levels. There were no essential differences between the responses of the hypertensive and the normal dogs.

In a second group of experiments propylene glycol and desoxycorticosterone acetate in propylene glycol were given intravenously to three hypertensive and three normal dogs. In twenty-six determinations no significant difference between normal and hypertensive blood pressure responses to the two preparations was noted. Two cubic centimeters of propylene glycol regularly produced a rise of 15-30 mm. Hg in both groups. The response was unchanged by the addition of 5 mg. of desoxycorticosterone acetate.

Aqueous desoxycorticosterone glucoside in dosage of 5 mg. intravenously had no pressor effect in any instance. In an acutely hypertensive dog anesthetized with pentobarbital sodium no significant pressor effects were observed with any of the three preparations.

38. INTRA-ARTERIAL BLOOD PRESSURE CURVES DURING TILTING

A New Method of Studying Hypertension

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Previous studies demonstrated that the blood pressure at the level of the aortic arch invariably rose during a tilt from 20 degrees erect to a 45-degree head down position and fell during the return tilt to the 20-degree erect position. These alterations were due to a change of the effect of the force of gravity on the central blood column.

These changes apparently acted as intra-arterial stimuli to blood pressure regulatory mechanisms. In normal subjects, maintained in the altered position, the primary elevation that occurred during the head down tilt was followed by a secondary reflex depressor response; the fall during the erect tilt by a pressor response, whereby the blood pressure returned within eight to eighteen seconds to approximately the initial level, regardless of position.

Similar studies have been completed in a series of fifty nonselected patients with hypertension. Primary mechanical alterations of blood pressure occurred during tilting as in the normal group. However, the secondary response to these alterations varied.

The elevation during the head down tilt was followed by four types of response:

1. Delayed depressor: the blood pressure rose further before falling.
2. Decreased depressor: the blood pressure remained at an elevated level for a prolonged period.
3. Normal depressor.
4. Increased depressor: the pressure shortly after the head down tilt was lower than in the semierect position. Patients with this type response usually complained of dizziness on bending over.

The fall during the erect tilt was also followed by four types of response:

1. Increased pressor: the blood pressure immediately surged beyond, then gradually returned to the initial level of the 20-degree erect position.
2. Normal pressor.
3. Decreased pressor: the blood pressure remained lower than the initial erect level for a prolonged period.
4. Delayed pressor: the blood pressure fell further before returning to the initial erect level.

Patients with the two latter types of response usually complained of dizziness on arising suddenly.

Various combinations of pressor and depressor response occurred in individual patients; that is, normal depressor with increased pressor, decreased pressor with normal pressor, etc. In a given patient the combination of response remained constant on successive tiltings.

In normal subjects the heart rate invariably slowed following the head down tilt. In some hypertensive patients the heart rate slowed as in the normal; in others it did not change.

It is hoped that this method of study of the cardiovascular reflexes will offer a means of classifying hypertension and indicate possible approaches to treatment.

39. ANTIBODIES AGAINST RENIN AND "SUSTAINED PRESSOR PRINCIPLE" PRODUCED BY INJECTIONS OF KIDNEY EXTRACTS

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Intramuscular or intraperitoneal injection of various kidney extracts into several animal species caused the appearance in the sera of a substance or substances capable of destroying or "neutralizing" renin and the "sustained pressor principle."

Extracts prepared from hog kidneys were administered over the period of a year to a series of ten hypertensive patients. Although antibodies against

hog renin and hog "sustained pressor principle" were formed in the serum of these patients, the antibodies failed to neutralize human renin or "sustained pressor principle." These extracts which did not possess irritating and shock-producing properties caused no lowering of blood pressure in the patients.

Of the several types of animals used, only the dog produced antibodies to its own renin when injected with heterologous kidney extracts.

40. EFFECTS OF SPLANCHNICECTOMY ON BLOOD PRESSURE AND ON CARDIAC AND RENAL FUNCTION

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One hundred eight hypertensive patients were studied before and ten to sixteen months after supradiaphragmatic splanchnicectomy (Peet). Thirty-one control patients acceptable for the operation who did not undergo surgery were re-examined ten to eighteen months later under similar conditions. These patients were comparable to the operated group in age, sex, height of blood pressure, and duration of hypertension and were treated by the usual medical methods, including mild sedation in some cases. Table I presents the significant differences in the observations on the two groups at the time of follow-up examination.

Table I

	POST SPLANCHNICECTOMY SERIES		CONTROL SERIES	
INITIAL STATUS OF PATIENT	NUMBER	% IMPROVED	NUMBER	% IMPROVED
Casual diastolic blood pressure exceeding 110 mm. mercury	80	21*	25	0*
Cardiac enlargement present by teleroentgenogram	15	26	5	0
Electrocardiographic abnormality present	81	22	9	0
Headaches	84	92	20	50
Irritability and nervousness	73	72	16	25
Dizziness	50	76	13	54

*More than 20 mm. decline in casual diastolic blood pressure at follow-up visit was considered "improved."

Only twelve of the patients had a diastolic blood pressure below 90 after operation. We believe, however, that there were definite evidences in certain of the other hypertensive patients that the operation improved their clinical status when they were compared with the control subjects. This result was accomplished with a minimum postoperative disability: patients left the hospital ten to twelve days after operation and were able to resume full activity in two to three months.

Sixteen of the splanchnicectomized patients who had had a 20 mm. decline in diastolic blood pressure were compared with seventeen patients who had experienced slight increases in pressure one year after operation. In general, the subjects in the group which improved were younger, had a higher initial blood pressure, contained more females, and showed a greater fall in blood pressure after tetraethylammonium than did those in the group showing poor results.

In thirty-four patients renal hemodynamics were studied before and at various time intervals after operation. Renal blood flow (para-aminohippurate clearance) was usually unchanged, but filtration rate (mannitol or thiosulfate

clearance) tended to fall, with a consequent decline in filtration fraction. In seven patients with a 20 per cent postoperative reduction in mean blood pressure, the renal blood flow was maintained, and there was a consequent decline in renal resistance suggesting some postoperative renal vasodilatation.

41. THE ARTERIOLES OF THE SKIN IN ESSENTIAL HYPERTENSION

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It is generally accepted that a diffuse disturbance of the arterial side of the vascular system exists in hypertension. Curiously enough, the cutaneous arteriolar beds in people with essential hypertension have not been studied adequately. For this reason we felt that a controlled study of the arteriolar bed of the skin in a group of persons who had essential hypertension might be of value.

Material for this study was obtained from the upper arm, lumbar region, and calf of fifty-two persons who had normal blood pressure and from seventy persons who had moderate to severe essential hypertension. The skin was obtained by means of excision and punch. All the material in the hypertensive group was secured from living people. The youngest person with hypertension was 29 years old; the oldest person was 80 years of age; the mean age was 44 years. Of the fifty-two specimens of skin removed from persons who had normal blood pressure, forty were taken from living people and twelve were obtained at necropsy.

Sections for microscopic study were made. A Bausch and Lomb micrometer eyepiece was used over a high-power objective, which produced a magnification of 430 times (43 × 10). With this instrument the average thickness of the wall of the vessel and diameter of the lumen were measured. We studied every slide from left to right and made measurements of the first four arterioles we saw in each slide.

A measurable thickening of the arteriolar wall and a decrease in the wall-to-lumen ratio as compared with normal were found in vessels of the skin of patients with hypertension. The average of the wall-to-lumen ratio of the arterioles of fifty-two persons with normal blood pressure was 1:2.14; among seventy persons who had essential hypertension it was 1:1.57. Not all arterioles were equally affected in the same case.

Qualitative changes were also present. Hyperplasia of the nuclear elements of the media and thickening of the inner elastic lamina appeared to be the most common changes. Occasionally, complete occlusion of the lumen occurred. Although the arterioles in the skin of persons who had malignant hypertension were more profoundly altered than were those of persons who had other types of hypertension, equally severe qualitative changes were present in the arterioles of a number of persons who had the other types of hypertension. Four patients with normal blood pressure had medial hypertrophy of the arteriolar wall.

42. RETINAL ARTERIOVENOUS NICKING

II. A LONG-TERM STUDY OF THE DEVELOPMENT OF ARTERIOVENOUS NICKING IN PATIENTS WITH HYPERTENSION

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We reported our studies on the significance of various stages of retinal arteriovenous nicking in patients with hypertension before this society in November, 1939. We have now re-studied a group of these same patients after eight-

to ten-year intervals and have followed for the first time the development of arteriovenous nicking from the earliest stages to the latest. We have thus shown for the first time that the so-called early lesion actually develops into the late lesion. The importance of distinguishing these lesions one from the other will be emphasized. Color drawings of unusual clarity will be presented showing the various types of arteriovenous nicking and other retinal arterial changes found in hypertensive patients.

43. THE INSULIN TOLERANCE OF THE HYPERTENSIVE PATIENT

Geza de Takats, M.D., and Jack Lasner, M.D. (By Invitation)
Chicago, Ill.

The insulin tolerance of fifty hypertensive patients have been studied. One-tenth of a unit of insulin per kilogram of body weight was injected intravenously and blood sugar levels were determined at 15, 30, 60, 90, and 120 minutes after each injection. A normal response was characterized by a drop of the blood sugar to about 50 per cent of the fasting level at thirty minutes followed by a return to normal in the next two hours.

In the present series of fifty patients, 16 per cent showed complete and 8 per cent showed a partial resistance to the intravenous dose of insulin. This insulin response was used to measure the presence of corticoadrenal activity. Such hyperactivity may be one of the causes of immediate failure following splanchnic nerve section, although delayed improvement may occur.

In addition, our previous observations that the insulin-resistant diabetic patient becomes insulin sensitive after splanchnic nerve section was observed in two patients with diabetes in this series.

44. PALPITATION

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Palpitation is a symptom usually much more alarming than serious. It consists of an unpleasant sensation of the heart's action whether slow or fast, regular or irregular. It more frequently does not indicate primary physical but rather a psychic disturbance.

Four hundred patients from Barnes Hospital, private practice, and the Washington University Clinics presenting various degrees of palpitation were studied to determine the clinical characteristics of this condition. Of this group, approximately half were clinic and the other half hospital patients. Sixty-four per cent were females and 36 per cent males. Palpitation was noted as a primary complaint in approximately half the entire group and as an accessory complaint in the remainder. The commonest age incidence was in the group from 40 to 50 years, and next in order of frequency were the sixth and fourth decades. One hundred ninety-five (49 per cent) had a severe form of palpitation, 183 (46 per cent) had this symptom to a moderate degree, and 22 (5 per cent) considered it mild. In women the complaint appeared more consistently in the severe form.

Of the 152 patients with cardiac and vascular disease, the principal etiologic factors included arteriosclerosis and hypertension (53 per cent), hypertension alone (18 per cent), and rheumatic fever (7 per cent). Seventeen per cent of the total group had extracardiac conditions of diverse type. The arrhythmias encountered in the 325 patients who had electrocardiograms in-

cluded ventricular extrasystoles (22 per cent), auricular fibrillation (12 per cent), sinus tachycardia (9 per cent), sinus bradycardia (6 per cent), and very few instances of auricular extrasystoles, auricular flutter, and paroxysmal auricular tachycardia. Two patients had complete heart block. Two hundred twenty-seven patients had basal metabolism tests; only three had rates above +30 per cent, and nine were in the range from +20 to +30 per cent. Only six patients had clinical hyperthyroidism.

Two hundred twenty-eight (57 per cent) of the entire group presented primary nervous complaints, and of these patients, 57 per cent had a definite anxiety state, 22 per cent were typical of neurocirculatory asthenia, 12 per cent presented prominent menopausal symptoms, and 9 per cent were psychotic. Analysis of the cases revealed no significant evidence that such factors as anemia, fever, digitalis, or smoking had any direct connection with this symptom. Excessive intake of alcohol or coffee seemed to produce marked palpitation. Undoubtedly the most common etiologic factor was the presence of a significant anxiety state, and when this was associated with emotional stress or fatigue, palpitation became not only a frequent, but also a most troublesome symptom.

45. OBSERVATIONS ON ONE HUNDRED PATIENTS WITH MYOCARDIAL INFARCTION WHO SURVIVED UP TO SIX YEARS

G. Y. Mills, M.D. (By Invitation), F. Cisneros, M.D. (By Invitation), and L. N. Katz, M.D., Chicago, Ill.

We have recently had the opportunity to make a follow-up survey on 100 patients who had survived an acute myocardial infarction for from one to six years.

Our patients represented a cross section of the population with varied occupations, interests, and income. Most of the patients had returned to their former jobs. In most instances the younger patients were more regularly able to resume a full share of previous activity after recovery than the older ones.

In general the trend was toward a higher blood pressure at follow-up than that which existed at the time of infarction. Males were more prone to have hypertension with the greatest incidence on the fifth decade. Hypertension was the most frequent finding among patients with little electrocardiographic restitution.

Angina pectoris occurred four times as frequently in males as in females, with the greatest incidence in the sixth decade. There appeared to be no relationship between angina pectoris and the amount of electrocardiographic restitution.

Heart failure was seen most frequently in patients in the sixth decade. Almost all of these patients showed little electrocardiographic restitution.

Combinations of angina, hypertension, and heart failure were most frequent among males than females, especially in the fifth and sixth decades. None of the patients with complete electrocardiographic restitution had a combination of angina, failure, or hypertension.

More than five times as many males as females were "asymptomatic"; the greatest incidence was in the fourth decade. Patients with previous lateral or atypical infarction electrocardiographic patterns were most likely to be asymptomatic. Approximately two-thirds of the asymptomatic patients had no disturbanes of conduction, rhythm, or voltage at the time of their infarction.

46. INTRAVENOUS CATHETERIZATION OF THE HEART IN THE DIAGNOSIS OF CONGENITAL HEART DISEASE

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Recent advances in surgical alteration or correction of certain congenital defects of the heart and great vessels make it imperative that more accurate diagnosis of such lesions be made. Intravenous catheterization of the heart has been a useful aid to ascertain the condition in patients suspected of having such congenital abnormalities.

A 6 French or 9 French special catheter is introduced into the median basilic vein and passed under fluoroscopic control via the subclavian vein and superior vena cava into the right side of the heart and into the pulmonary artery and its branches. The oxygen content of samples of blood are obtained at various sites and should not differ normally by more than 1.9 volumes per cent. Pressures in the various sites are recorded by means of a Hamilton manometer.

This procedure has been used in 58 patients with, or suspected of having, congenital defects of the heart or great vessels. The results illustrate its value to ascertain whether or not the patient has a defect, to indicate the operability when defects are discovered, and to suggest the prognosis.

In cases of auricular septal defect the oxygen content of the blood from the right atrium is greater than that in the vena cavae. In patients with ventricular septal defects, a significant increase in oxygen content in the blood from the right ventricle is demonstrated when compared with that in the atrium. Septal defects may also be demonstrated by passing the catheter through the defect into the left side of the heart. Patent ductus arteriosus is diagnosed by finding an increase in oxygen content of blood from the pulmonary artery as compared with the right ventricle, and occasionally by an increased pulmonary arterial pressure. Cases of cyanotic congenital heart disease are presented, showing the usual absence of left to right shunt. Cyanotic patients with increased pressure in the right ventricle or pulmonary artery are discussed. Combined lesions such as cyanotic congenital heart disease with patent ductus arteriosus may be found and patients with such abnormalities are described. Several patients who were suspected of congenital defects but in whom the catheterization studies were normal are described. A case in which a pulmonary vein was found to empty into the right (?) or common (?) atrium is reported. Complications of the procedure, including premature ventricular contractions, phlebitis, and venospasm, are discussed.

47. DIETARY AND HORMONAL INFLUENCES IN EXPERIMENTAL ANURIA

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IRVINE H. PAGE, M.D., CLEVELAND, OHIO

The importance of diet in the treatment of uremia has been recently emphasized by Borst. The present study was devised to test experimentally the effects of high iso-caloric (9 Cals. per 100 sq. cm. body surface per day) carbohydrate, fat, and protein diets and of the provision of hormones (desoxycorticosterone acetate and free testosterone, 5 mg. daily of aqueous suspension) on survival times and azotemia (blood urea N) in bilaterally nephrectomized rats. All treatment was begun at the time of nephrectomy.

Data are summarized in Table I. Survival was definitely improved and azotemia greatly diminished by administration of fat or carbohydrate rather than protein. Survival was somewhat more prolonged and azotemia decreased by administration of carbohydrate rather than fat.

TABLE I. MEAN SURVIVAL TIMES AND BLOOD UREA NITROGEN LEVELS IN BILATERALLY NEPHRECTOMIZED RATS

DIET	TREATMENT	NUMBER OF ANIMALS	SURVIVAL TIME (HR.)	BLOOD UREA NITROGEN (MG.)	
				AT 30 HR.	AT 48 HR.*
Protein	0	40	42	264	550
	DCA	10	44	263	543
	Testosterone	10	44	240	461
	DCA and testosterone	10	52	281	510
Carbohydrate	0	20	93	112	134
	DCA	9	127	89	131
	Testosterone	9	118	100	130
	DCA and testosterone	9	116	60	90
Fat	0	10	74	135	175
	DCA	9	79	113	167
	Tesotsterone	9	74	121	173
	DCA and testosterone	9	71	122	166

*In Survivors.

Treatment with hormones had no significant effect on survival time regardless of diet, and little effect on azotemia. The data indicate a decrease in blood urea in rats treated with DCA and testosterone on the high carbohydrate diet. It is not established that this change is significant and it is, in any case, evident that it does not improve survival.

These experiments substantiate the beneficial effects claimed by Borst for the treatment of uremia by administration of a high caloric diet, composed largely of carbohydrate. They do not confirm the suggestions of others that hormonal treatment is of value after nephrectomy.

48. PLASMA PROTEIN STUDIES IN CHILDREN WITH RHEUMATIC FEVER

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Recent statistical studies completed by this clinic revealed an unexpectedly low recurrence rate for a highly susceptible group of young rheumatic children who had received special attention to improve their diets and level of home care. Prior to management, the diets of these children commonly were found to be deficient in protein. The relation between analysis and course of the disease was studied. In general the electrophoretic partition between the albumin and the total globulin fractions confirmed the chemical analysis. The fibrinogen fraction tended to be higher by the electrophoretic method. During the more active phase of rheumatic fever the values for plasma albumin were consistently and markedly decreased, while the values for gamma globulin, alpha$_2$ globulin, and fibrinogen were increased. The changes in the gamma globulin fraction were particularly pronounced. With improvement in the clinical condition of the child, the distribution of the plasma proteins shifted toward the normal. Return of the sedimentation rate and the plasma albumin to normal values generally occurred simultaneously, but exceptions were observed.

49. THE RELIEF OF EDEMA IN NEPHROSIS BY PLASMA ALBUMIN AND GELATIN INFUSIONS

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Seven patients with advanced nephrotic edema, who had been treated by the usual methods without benefit, were given large amounts of salt-poor plasma albumin intravenously. There were two failures, one of which was a death from infection. Four cases progressed to a satisfactory remission after the marked diuresis which was obtained and one case is still pending. Three patients have been diuresed most satisfactorily with 10 per cent gelatin in distilled water, and three have been treated with both gelatin and albumin with very satisfactory results. These experiences strongly suggest that a greatly hastened remission in cases of the nephrotic syndrome is often possible because of the effective diuresis brought about by this method.

50. THE ACTION OF SEVERAL CARDIAC GLYCOSIDES ON EXCITABILITY AND CONDUCTION VELOCITY IN THE DOG HEART

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Digitoxin (Digitaline-Nativelle), lanatoside C (Cedilanid), ouabain (Arnaud), and k-strophanthoside (Strophosid) were studied in dogs under chloralose anesthesia. The heart was exposed and electrodes attached at various points on the surface of the right auricle and both ventricles, to permit measurement of A-V conduction, linear conduction on the surface of the ventricles in response to electrical shocks, and the threshold of electrical excitability of both auricle and ventricle. The glycosides were injected at thirty-minute intervals in doses calculated to kill the heart in about four hours.

Auricular excitability began to diminish after the first dose, reaching 50 per cent of normal at 55 to 70 per cent of the lethal dose. Auricular arrest occurred at about 80 per cent of the lethal dose, when auricular excitability was from 17 to 45 per cent of normal. Shortly thereafter the auricles became completely inexcitable.

Ventricular excitability increased slightly (10 to 20 per cent) after the first dose, but after the administration of 60 to 80 per cent of the lethal dose began to diminish rapidly, reaching levels of 50 to 60 per cent of normal shortly before death. Although the first idioventricular beats were recorded at a time when the ventricle was more than normally excitable, the frequency of extrasystoles increased, leading eventually to multifocal ventricular rhythms and fibrillation, as the excitability diminished below normal levels. The ectopic activity produced by toxic doses of digitalis cannot therefore be explained in terms of electrical excitability but rather of increased "automatism."

A-V conduction, as expected, was slowed progressively after the first dose, until complete block occurred at 55 to 70 per cent of the lethal dose. Severe impairment of A-V conduction was evident before any slowing of intraventricular conduction could be measured. Ventricular conduction from a stimulating to a recording electrode less than 15 mm. distant was rarely delayed and often accelerated even in the terminal stage of intoxication. The time interval between near and distant electrodes was not altered until about 70 per cent of the lethal dose had been administered, but then increased progressively until death. Since propagation of a forced beat from the point of stimulation to a near electrode must represent chiefly muscular conduction, and to a distant

point chiefly Purkinje conduction, it would appear that digitalis specifically depresses the specialized conducting tissue. Digitalis fibrillation becomes possible when conduction in the Purkinje tissue has been depressed so much that an excitation wave has not yet reached the most distant fibers at the time when a new impulse has begun.

51. ETIOLOGY OF AURICULAR FIBRILLATION AND THE MECHANISM OF ITS PERPETUATION

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(Introduced by L. N. Katz, M.D.)

Observations on man and the dog are presented which relate to the etiology of auricular fibrillation and the mechanism of its perpetuation. Vagal stimulation and anoxia are the main etiologic factors in the initiation and perpetuation of auricular fibrillation.

Vagal stimulation (mechanical and chemical) may induce auricular fibrillation. Acetylcholine injected directly into the blood stream was used in our experiments to produce chemical vagal stimulation. Moderate anoxia reduces the threshold of the auricles to the initiation of fibrillation but does not induce this arrhythmia per se; marked anoxia, on the other hand, increases the threshold to initiating fibrillation.

Anoxia of the auricles was found or produced (1) by interference with or obstruction of its vascular supply, (2) by a decrease in the amount of oxygen carriers, (3) by a decreased oxygen content of the blood due to anoxic anoxemia, and (4) by interference with tissue respiration. The relationship between vagal stimulation and anoxia can be plotted in a graph and on this correlation the cause of the perpetuation of auricular fibrillation can be demonstrated. The clinical implication of these findings and the therapeutic approach to this problem are illustrated and discussed.

52. DICUMAROL AND QUINIDINE IN THE AMBULATORY TREATMENT OF CHRONIC AURICULAR FIBRILLATION

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This report consists of a study on thirty-three cases of chronic auricular fibrillation treated from the outpatient department of the Los Angeles County General Hospital.

The purpose of this investigation was to (1) add preventive measures against possible thrombus formation in the auricle and thereby help eliminate the probability of embolic phenomena after restoring a chronic fibrillating heart to normal rhythm; (2) to re-establish normal sinus rhythm; (3) To re-evaluate the difference between the cardiac output in a slow fibrillating heart and that of the heart after it has been restored to normal sinus rhythm.

Material.—

Sex: Twenty-nine males; four females.

Age range: 17 to 83 years.

Etiology: Rheumatoid group, twenty-one cases; arteriosclerotic and hypertension group, twelve cases.

Duration of fibrillation: Approximately two years.

Technique.—(1) Patients were first digitalized. (2) Circulation time, venous pressure, and vital capacity were then determined. (3) Dicumarol was administered until a prothrombin level of between 30 and 20 per cent was obtained. (4) Quinidine therapy was then given in daily increasing doses. The maximum amount of quanidine given any patient in a twenty-four hour period was 30 grains. (5) After normal sinus rhythm was obtained, circulation time, venous pressure, and vital capacity were again determined.

Results.—In eighteen of the thirty-three patients, 55 per cent, normal rhythm was restored. It was also restored in ten patients, 48 per cent, of the rheumatoid group and eight, 67 per cent, of the arteriosclerotic and hypertension group.

Laboratory findings in the restored group: *Vital capacity* increased on the average of 20 per cent. *Venous pressure* lowered on the average of 60 mm. *Circulation time:* ether, increased on the average of 3 seconds. Calcium gluconate, increased on the average of 8 seconds.

53. EXPERIMENTAL STUDIES ON THE COMBINED ACTION OF DIGITALIS (CEDILANID) AND QUINIDINE (IN CATS)

S. A. Weisman, M.D., Los Angeles, Calif.

The meaning of the term "combined action" of quinidine and digitalis on the heart, as discussed in the literature, perhaps warrants some clarification. It usually refers to the action of these drugs on the heart after they have been given following each other at varying time intervals.

The purpose of this study was to (1) reinvestigate the pharmacologic action of digitalis and quinidine on the heart after giving these drugs in consecutive order at varying time intervals; (2) to study the effect of these drugs on the heart after both drugs are administered together, and (3) to reinvestigate the effect of quinidine on respiration following the administration of digitalis and the effect on respiration after these drugs are given simultaneously.

Comparative studies were made on the (1) mortality, (2) heart rate, (3) blood pressure, (4) electrocardiographic changes, (5) respiration.

Results.—It was found that there appeared to be less toxic effects on the heart and the respiration after these drugs were administered together than when they were given in consecutive order.

The clinical application of these observations is discussed.

54. PAROXYSMAL TACHYCARDIA, LOW NODAL IN ORIGIN, BENEFITED BY CARDIAC SYMPATHECTOMY

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A 35-year-old, cyanotic, white female, a complete invalid because of incapacitating paroxysms of tachycardia lasting up to fifteen minutes in duration and occurring thirty to forty times per day, was admitted to the hospital for study.

General medical history was of cyanosis since birth, rheumatic fever at 6 years of age, diphtheria at 7, mastoidectomy at 7; at 22 years she had had a cerebrovascular accident with hemiplegia and aphasia, which essentially cleared in six months.

The exact nature of the congenital cardiac anomaly was not determined, but it was clinically diagnosed as cor triloculare biatriatum. The laboratory work-up revealed a slight polycythemia (hematocrit, 55 per cent; R.B.C., 5.5 million) and electrocardiographic evidence that the origin of the impulse of the cardiac stimulus was either low nodal or high in the bundle of His.

The first attack of paroxysmal tachycardia noted by the mother was when the patient was 3½ years of age, and the attacks had progressed from once a month to the present frequency. It was early noted that vomiting caused a cessation of the fast rate and this and other manipulations were utilized by the patient. Electrocardiographic tracings were taken when the patient would initiate a paroxysm of tachycardia by exertion and then stop the same by pharyngeal stimulation. Cardiac asystole up to 3 seconds in duration followed this conversion of rhythm.

It was surprising that the autonomic nervous system exercised control over cardiac impulse of such low origin. To establish the presence of this control a pharmacologic approach was taken, yielding the following results: Atropine sulfate, given by hypodermic needle, within ten minutes produced a tachycardia which persisted for several hours; quinidine sulfate in minimal doses resulted in an increase in frequency and duration of the paroxysms of tachycardia; Prostigmine produced no change; digitoxin in therapeutic and near toxic doses gave a decrease in frequency and duration of the paroxysms; di-isopropyl-fluorophosphate resulted in decreased frequency and duration of the paroxysms. (Di-isopropyl-fluorophosphate destroys cholinesterase which in turn allows enhancement of the vagus through prolonged action of acetylcholine.)

In the light of these findings it was felt warranted to remove the cardiac autonomic accelerators (the cardiac sympathetics) in an attempt to stabilize the cardiac rhythm at its slower rate. A two-stage bilateral cardiac sympathectomy of stellate ganglion and the first, second, third, fourth, and fifth thoracic nerves was done.

Since surgery the patient has experienced only about five paroxysms of tachycardia per day, lasting only a few seconds.

55. THE EFFECT OF APPREHENSION CAUSED BY THE TECHNICAL PROCEDURES ON CARDIAC OUTPUT

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In the course of studies of the effect of spinal anesthesia on cardiac output, the variability due to apprehension was so extreme as to make the results of these studies wholly unreliable because initial control values within normal basal range could seldom be obtained. Furthermore the cardiac output varied unpredictably during the course of the procedures. The variations from standard normal values were always high.

This report is based on a study of cardiac output by the direct Fick method in twenty-four individuals. There were never less than three serial determinations of cardiac output during an experimental period in any patient. In nine patients four consecutive cardiac outputs were done at each experimental period.

It was our belief that high values were due to apprehension. In general, the rate of oxygen consumption bore out this belief. Attempts to use the appearance of the patient, pulse rates, and respiratory rates as criteria for excitement failed to correlate with either our cardiac output results or with the oxygen consumption.

When control values of cardiac output were within normal limits, spinal anesthesia to the first thoracic nerve did not cause decrease in cardiac output. When control cardiac output values were high, spinal anesthesia caused them to drop to normal levels. This is probably due to temporary denervation of the heart caused by the high spinal anesthesia.

Our frequent inability to obtain normal cardiac output values for control observations caused us to sedate our patients with Nembutal, morphine, and scopolamine prior to and during the observations. The uniformly normal control cardiac outputs which we obtained in twelve patients with such sedation have been so satisfactory as to make us believe that sedation is essential in studies which are directed at determining the effect of certain procedures on cardiac output.

56. THE EFFECTS OF ACUTE HYPOXIA ON THE SENSITIVITY OF THE HEART TO ACETYLCHOLINE

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This laboratory has recently been engaged in a study of the physiologic responses of the circulation to acute hypoxia. We have shown that during acute severe hypoxia the response of the blood vessels to epinephrine is greatly reduced and that this substance persists in the blood beyond the hypoxic period when relief of hypoxia is quickly introduced. The effect of hypoxia on the action of acetylcholine on the cardiac pacemakers has also been analyzed. In the present study the effect of hypoxia on the sensitivity of the heart to acetylcholine was measured.

In nine dogs catheters were inserted into the left femoral artery and passed under fluoroscopic control into the region of the sinuses of Valsalva. One hundred two injections of acetylcholine were used during hypoxia and the decrease in rhythmicity and conductivity which was produced was recorded electrocardiographically. The threshold dose of acetylcholine injected via the catheter required to produce slowing or block was established during air breathing before and after each hypoxia test. Nitrogen breathing was substituted for air to produce the hypoxia. It was found that the sensitivity of the heart to acetylcholine increased progressively with the continuation of nitrogen breathing, as illustrated in Table I.

Table I

DURATION OF HYPOXIA (SEC.)	INCREASE IN R-R DISTANCE (SEC.)	PRESENCE OF A-V BLOCK
Control	0	0
30	0.3	0
60	0.5	+
100	1.1	+
140	1.7	+
180	2.5	+
220	3.8	+

Relief of hypoxia by reoxygenation resulted in a return of the acetylcholine sensitivity to normal within the next minute or two.

The cardiac slowing and asystole sometimes seen in acute hypoxia may be related to a similar potentiation of acetylcholine action. It is possible that the apparent increase of cardiac sensitivity to acetylcholine is in reality the result of continued presence during hypoxia of endogenous acetylcholine in increasing amounts summating with that injected.

57. THE EFFECT OF CERTAIN SYMPATHOLYTIC AGENTS ON THE CORONARY BLOOD FLOW OF THE DOG

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The effect of tetraethylammonium bromide and Dibenamine on the coronary blood flow of dogs was investigated using a modified Morawitz cannula technique for measuring the coronary sinus outflow. The conditions of administration approximated those used in ordinary clinical practice. With tetraethylammonium bromide there was no influence on the coronary sinus outflow while the drug was being injected. After the intravenous infusion was completed, a small increase of the flow was usually observed. The drug had no effect on the characteristic coronary vasodilator action of adrenalin. Intramuscular injection of tetraethylammonium bromide had no detectable influence on the coronary blood flow over a period of several hours. When Dibenamine was given by venoclysis, no change was seen in the coronary flow during, or for several hours after, administration. With the doses used the peripheral vasopressor action of adrenalin was either reversed or nullified. The typical coronary vasodilator action of adrenalin was not affected by Dibenamine. In dogs that had received Dibenamine, stimulation of the peripheral ends of the cut vagi resulted in a modified response. It is apparent that the action of these sympatholytic agents on the coronary blood flow is distinctly different from their action on the blood flow in the extremities. Since these animals were all normotensive it was not surprising to find that there was only a moderate decrease in systolic blood pressure during the administration of tetraethylammonium bromide and Dibenamine. The fact that these sympatholytic agents which are capable of markedly increasing the blood flow of the limbs did not alter the coronary blood flow is of clinical interest.

58. DIBENAMINE

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A study was made of the effect of Dibenamine (dibenzyl-beta-chloroethylamine) on blood pressure and the peripheral arterial system. Toxic effects were noted. The drug is a tertiary amine and is sympatholytic and adrenolytic in action.

Dibenamine was given intravenously to six patients. Five of the patients had essential hypertension and one had normal blood pressure. Skin temperatures were determined on four of the patients after the administration of Dibenamine. The first patient studied received a dose of 2.5 mg. per kilogram and the other five received a dosage of 4.0 mg. per kilogram.

Dibenamine caused a marked orthostatic hypotension lasting from five to twenty-four hours in all six patients. It also caused a rise in the skin temperature of the lower extremities. The maximum rise in temperature occurred within three to five hours after giving the drug and lasted for from five to forty-nine hours. The vasodilator effect upon the skin lasted longer than the effect upon the blood pressure. Toxic reactions consisted of nausea and vomiting in one case and thrombophlebitis in another. Marked vertigo and faintness occurred in all patients on assuming the standing position. These symptoms disappeared upon reclining. Contraction of the pupils occurred in all. Nasal congestion occurred in five, and two patients complained of dryness of the mouth.

59. THE EFFECTS OF DIHYDROERGOCORNINE ON THE PERIPHERAL CIRCULATION IN MAN

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ROCHESTER, MINN.

The effects of the intravenous administration of dihydroergocornine (DHO-180), an ergotoxic alkaloid, on skin temperatures, blood pressure, heart rate, and peripheral blood flow were studied in twenty human volunteers. The action of the drug is chiefly sympatholytic and, therefore, the drug is considered to be a vasodilator. It was administered to six patients by intravenous infusion in a solution containing 0.5 mg. of dihydroergocornine per 100 c.c. of physiologic saline solution and to fourteen patients by a single intravenous injection. The total dosage varied from 0.25 to 0.4 mg. Control values for skin temperatures, blood pressure, heart rate, and blood flow were determined before the drug was given, and the observations were again recorded at regular intervals for a period averaging sixty-five minutes after administration of the drug. The blood flow was determined by means of a venous occlusion plethysmograph with a compensating spirometer recorder. Skin temperatures were recorded by means of thermocouples applied to the skin over the forehead, over the right and left deltoid muscles, and over the right and left quadriceps femoris muscles.

Dihydroergocornine produced an over-all average increase in peripheral blood flow of 95 per cent in the upper extremities and 68 per cent in the lower extremities in nineteen of twenty cases. In spite of the increase in blood flow in the extremities, the skin temperatures were slightly decreased, even during the maximal increase in blood flow.

The blood pressure fell in the two hypertensive patients after administration of dihydroergocornine. The decrease of systolic pressure was 58 mm. Hg in one case and 30 mm. Hg in the other, while the diastolic pressure fell 18 mm. Hg in the former and 10 mm. Hg in the latter. In normotensive subjects there was no significant change in blood pressure.

The heart rate decreased in every case, with an average reduction of 13 beats per minute.

Side reactions were more frequent than had been reported by other investigators, even with lower dosage. Nasal congestion, nausea, headache, flushing, an urgency for urination, and vomiting were the side reactions observed.

60. RELATION OF TOBACCO SMOKING TO DISEASES OF THE RESPIRATORY AND CIRCULATORY SYSTEMS

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In order to have a satisfactory picture of *normal* smoking habits, we made a house-to-house survey in every census tract of the city of Columbus, Ohio, with a verbal questionnaire on individual smoking habits of persons over 20 years of age. About 1,000 persons of each sex and color were interviewed and the results summarized by age groups and city districts (suburban, intermediate, and slum). The details of this survey will serve as a useful basis of normal smoking habits in future studies, when they are published in detail.

The smoking habits of persons dying of buccal and respiratory tract cancer in Cincinnati and Detroit were compared with those of the Columbus controls and the following differences noted: (1) The incidence of cigar and pipe smok-

ing was very significantly higher among both the buccal and the respiratory tract male cancer victims than among men of corresponding ages in Columbus, while the incidence of nonsmokers was significantly lower than among the proper Columbus controls. Cigarette smoking incidence did not differ significantly from the Columbus standard for either these male or female cancer victims.

With individuals suffering from pulmonary tuberculosis in Dunham Hospital (Cincinnati), the pipe and cigar smoking habits of men were found to be similar to those of the proper Columbus controls, but the incidence of cigarette smoking was significantly higher than normal among both male and female patients. Studies on smoking habits of pneumonia victims are not yet far enough along to report.

Although tobacco smoking has been strongly indicted in various diseases of the circulatory system, adequate detailed and convincing proof has been lacking up to now. To supply proof which could be quantitatively essayed on a mathematical basis, we mailed questionnaires on tobacco smoking habits to the next of kin or informant named on death certificates of all white persons dying in Cincinnati from coronary heart disease during the two years 1946 and 1947. From the returned questionnaires the following findings were obtained: (1) all white victims under 40 years of age were cigarette smokers; (2) the heavy preponderance of cigarette smokers gradually decreased to near the expected normal by the age of 70 years, but by then pipe and cigar smoking was significantly above normal; (3) at all ages the percentage of nonsmokers was very significantly below that found among appropriate Columbus controls.

This survey is being extended to include peptic ulcer and certain other states in which nicotine effects on the autonomic ganglion cells might play a role.

It does seem clear that cigar and pipe smoking is significantly related to cancer of the air passages and lungs, while cigarette smoking is significantly related to infectious diseases of the respiratory tract. Cigarette smoking below 65 years of age and pipe or cigar smoking above that age are significantly related to fatal attacks of coronary heart disease.

61. FACTORS INFLUENCING THE T WAVE OF THE ELECTROCARDIOGRAM; AN EXPERIMENTAL STUDY EMPLOYING INTRACAVITARY AND EPICARDIAL LEADS

I. Effects of Heating and Cooling of Subepicardial and Subendocardial Lamina

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(Introduced by Harold Feil, M.D.)

Only recently has the question been raised as to the contribution of depolarization and repolarization of the subendocardial lamina to the genesis of the electrocardiogram. The employment of intracardiac catheter electrodes has facilitated the study of the electrical influence of the myocardium. In the present study this technique was employed to investigate the genesis of electrical events established by changes in the rate and direction of repolarization of subepicardial and subendocardial lamina. These changes were produced by heating and cooling the epicardium and the endocardium. Intra- and extraventricular (epicardial) exploring unipolar electrodes were used. The method of changing the temperature of the subendocardial surface relative to that of the epicardium consisted of introducing physiologic saline at various temperatures through indwelling intracavitary catheter electrodes. Epicardial thermal changes were

produced by local application of cold and hot saline-soaked pads and ice cubes of various sizes. The effects were immediate, with rapid recovery occurring in ten seconds to two minutes.

Negative T waves occurred where the exploring electrode immediately subtended areas which had relatively or absolutely retarded repolarization; positive T waves occurred where the exploring electrode faced areas which had relatively or absolutely accelerated repolarization.

It was found that negative T waves occurred:

A. In the cavity (1) when the endocardium was cooled; (2) when the endocardium was not altered, but the epicardium was heated.

B. On the epicardium (1) when the epicardium immediately under the electrode was cooled; (2) when the endocardium subjacent to the epicardial exploring electrode was heated; (3) when the endocardium of the opposite wall was cooled.

Positive T waves occurred:

A. In the cavity (1) when the endocardium was heated; (2) when the epicardium was cooled.

B. On the epicardium (1) when the subjacent epicardium was heated; (2) when the subjacent endocardium was cooled.

Direct evidence has been obtained that changes in the electrical state of the subendocardial muscular lamina modify the form of the electrocardiogram. Changes in the T wave can be produced by changing the rate and order of endocardial-epicardial laminar repolarization.

The relation of our results to the modern concept of altered repolarization in an extensive conducting medium is discussed. The spatial relation of the exploring electrode to a theoretical surface at the junction of the normal and altered areas is used to account adequately for the findings.

The practical value of these observations is discussed.

The present results confirm and extend previous observations on the contribution of the subendocardial myocardium to the electrocardiogram.

62. A DIRECT WRITING OSSILOGRAPH FOR RECORDING DATA OBTAINED BY THE METHOD OF CARDIAC CATHETERIZATION

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With the cooperation of the Brush Development Company of Cleveland, we have adapted their direct-writing six channel Ossilograph for recording simultaneously such data as the electrocardiogram, femoral arterial pressure, intracardiac or pulmonary arterial pressure, the respiratory cycle, time in seconds, and the Ballistocardiogram. By using strain amplifiers, pressure transmitters, and strain gauges we have found that this system records accurately variations in blood pressure as checked with the Hamilton manometer. Furthermore the system has certain advantages such as linearity of range scale, mobility, and direct visualization of phenomena being recorded and it is simple to calibrate. Data as recorded by the Ossilograph and derived from observations on patients with heart disease are presented in a series of lantern slides.

63. A COMPARATIVE EVALUATION OF EXTREMITY AND PRECORDIAL LEADS IN THE DIAGNOSIS OF ACUTE MYOCARDIAL INFARCTION

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(INTRODUCED BY EDMUND F. FOLEY, M.D.)

There has been a great deal of controversy concerning the relative merits of standard leads I, II, and III versus extremity potentials aV_R, aV_L, and aV_F. In addition the decision whether to use a precordial position with CF, CR, or V as the indifferent electrode has not been decided. Our purpose was to evaluate and compare these leads in recent acute myocardial infarction.

Fifty patients were studied, of whom twenty-five were shown by serial electrocardiographic, clinical, and autopsy data to have had an acute myocardial infarction. Of these twenty-five patients, seven showed equal diagnostic findings in standard leads, extremity potential leads, and precordial leads. Six had anterior involvement and one posterior. There were five in whom the precordial leads showed the most significant findings. The extremity leads I, II, and III and aV_R, aV_L, and aV_F were not normal but showed no acute involvement as manifested by S-T segment elevation or T-wave inversion. In five the extremity potentials aV_R, aV_L, and aV_F were more helpful in making a diagnosis than were the standard leads. In these the precordial leads were very useful also. Four patients with posterior infarctions showed the best changes in the extremity leads, precordial leads not being of much aid. Four others with posterior involvement were definitely aided by precordial leads.

The second study concerning the use of the left foot, the right arm, or the central terminal as the indifferent electrode position did not reveal significant differences. The voltages were not the same, but for clinical purposes they so closely resembled one another that we felt one indifferent electrode position to be as useful as another. When an S-T segment was elevated or a T wave inverted or a deep Q wave present in CF_4, for example, it was similarly altered in CR_4 and V_4.

In conclusion the extremity potentials aV_R, aV_L, and aV_F show diagnostic changes more frequently than the standard leads I, II, and III. No significant clinical differences were seen between CF, CR, or V precordial leads in the diagnosis of acute myocardial infarction.

64. THE ELECTROCARDIOGRAPHIC RESPONSE TO THE ANOXEMIA TEST IN PATIENTS WITH HYPERTHYROIDISM

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Observations of an anoxemia test that had been performed on a patient taking thyroid extract revealed a positive test. The test became normal on discontinuing thyroid. This led to the study of nine patients with clinical hyperthyroidism. All nine patients were selected carefully so that the symptoms were of recent duration; the basal metabolic rates on repeated tests were over 30 per cent, the age was under 40 years, and repeated examination revealed no evidence of cardiac disease.

It was then presumed that we were dealing with individuals whose hearts were normal except for any influence the hyperthyroidism might have had.

In all nine patients positive tests were observed. The S-T segments became depressed below 1 mm. and T-wave inversion especially in Leads I, II, CF_4 and V were seen. As the hyperthyroidism was controlled by medical management, propylthiouracil or surgery, the response to the anoxemia test was not as dramatic. Further studies are in progress at this time.

This test with the production of anoxemia has shown objectively the damaging effect of the hyperthyroidism. By implication one might anticipate that patients with normal thyroid glands taking thyroid extract for purposes of reduction would show similar changes.

65. ELECTROCARDIOGRAPHIC CHANGES FOLLOWING MEALS IN PATIENTS WITH ANGINA PECTORIS

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

Standard mixed meals, consisting of 90 grams carbohydrate, 40 grams protein, and 40 grams fat were given to thirty-two patients with angina pectoris. Electrocardiograms were taken on all patients before meals and thirty minutes following the meal. In some cases electrocardiograms were also taken sixty minutes and ninety minutes following meals. Warm food and warm liquids were used in each meal. In approximately 25 per cent of the patients there occurred a ½ to ¾ mm. depression of the S-T segment and development of, or increase in, existing concavity of the S-T segment. In patients showing this S-T change following meals, repeat electrocardiograms showed these findings to be consistent. Fourteen normal controls in the same age group exhibited no similar change.

The effect of meals on the electrocardiogram in these normal controls was similar to that reported by Simonson, Alexander, Henschel, and Keys in normal individuals. These changes consisted of the following: a significant increase in heart rate, decrease in Q-T interval, decrease in amplitude of the T wave, increase in QRS amplitude, and shift of the electrical axis toward the left. The patients with angina pectoris exhibited these changes also.

The electrocardiogram changes induced by the ingestion of food in the anginal patient suggests the possibility of employing this or similar methods as an additional aid in detecting coronary insufficiency.

66. CIRCULATION TIMES FOR ANGIOCARDIOGRAPHY

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(INTRODUCED BY DON C. SUTTON, M.D.)

The diagnostic justification for the performance of angiocardiography frequently depends upon obtaining a satisfactory levoangiocardiogram. This has been difficult to do when using the method of Robb and Steinberg because of difficulty in determining in advance when the contrast media will fill the left heart and aorta. Previous methods of determining circulation times prior to angiocardiography have not been totally satisfactory. Experiments in circulation time conducted by our group confirmed the work of others who had noted that increasing the volume injected decreased the circulation time. In a

group of twenty adults, none of whom had valvular cardiac lesions, increasing the volume of saline diluent from 5 to 50 ml. decreased the ether arm to pulmonary capillary circulation time by a mean of 1.3 seconds. A similar increase in volume decreased the sodium cyanide arm to carotid sinus circulation time by a mean of 6.3 seconds. The increased volume of material rapidly injected into the arm vein accelerated the circulation time *to* the right heart and *through* the lungs and left heart as well. Angiocardiograms timed on the basis of circulation times determined by the use of quantities of material equal to the contrast media used (50 ml. in this series), and injected in a manner identical to the media, produced satisfactory levoangiocardiograms.

67. A COMPARATIVE STUDY OF PERIPHERAL AND DIRECT INTRACARDIAC ANGIOCARDIOGRAPHY

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(INTRODUCED BY DON C. SUTTON, M.D.)

Two methods are available for the performance of angiocardiography. The first method, utilizing the injection of the contrast media into a peripheral arm vein, fails to provide satisfactory visualization in a number of patients. This occurs most frequently in adults who possess thick, muscular thoracic walls or dense mediastinal or lung lesions which lessen the contrast of the dye-filled structures. Direct intracardiac injection of the contrast media by means of a catheter introduced into the jugular vein in the neck and advanced to the right auricle is the second method. A comparative study of these two procedures has been made by the group working in Preble Laboratory.

68. THE APPEARANCE OF NORMOBLASTS IN THE PERIPHERAL BLOOD IN PATIENTS WITH PULMONARY EMBOLISM

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

Greater attention is being directed toward the study of the peripheral blood in cardiac failure and associated conditions. The quantitative changes, including the cellular elements, have been observed and reported. References to the qualitative changes of the cellular elements are extremely infrequent. During the past two years we have carefully studied the peripheral blood of all cases of pulmonary embolism that have come to our attention. We are reporting six cases of pulmonary embolism or thrombosis, with resultant right heart failure and the appearance of normoblasts in the peripheral blood stream. Other less noteworthy qualitative changes were observed, such as the findings of reticulocytes and polychromasia. All cases were autopsied, three of which showed extramedullary hematopoiesis. The explanation for the outpouring of normoblasts is not known. Acute severe anoxia would seem to be the most plausible etiologic factor. However, the anoxia produced by sudden altitude changes has not been reported to release normoblasts to the peripheral blood. In view of this, it is felt that other factors must play a role. Some of the additional factors postulated include tissue destruction and hemolysis.

(To be concluded in the December issue.)